Exploring the protective role of apocynin, a specific NADPH oxidase inhibitor, in cisplatin-induced cardiotoxicity in rats

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Abstract

Despite the clinical reports, few studies have focused on reducing the cardiotoxicity of cisplatin. In the present study, cardiotoxicity was examined after a single ip injection of cisplatin (7 mg/kg) in rats. Apocynin was given in drinking water (600 mg/L) for five successive days before and after cisplatin injection. At the end of the experiment, hemodynamic parameters were recorded, animals were sacrificed and serum creatine kinase-MB activity was determined. The whole ventricle was isolated for estimation of tumor necrosis factor-alpha (TNF-α) content, NADPH oxidase, myeloperoxidase and caspase-3 activities in addition to nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1) and nuclear factor kappa B (NF-κB) gene expressions. Furthermore, oxidative stress markers and antioxidant enzymes were measured in postmitochondrial and mitochondrial fractions. Mitochondrial membrane potential, nuclear DNA fragmentation and cardiomyocyte cross-sectional area were also evaluated. Apocynin was effective against cisplatin-induced decrement in heart rate and blood pressure. Moreover, pretreatment with apocynin notably ameliorated the state of oxidative stress, mitigated inflammation and preserved mitochondrial membrane potential. Apocynin provided also a significant cardioprotection as revealed by alleviating the overexpression of Nrf2, HO-1 and NF-κB, the elevation of caspase-3 activity, the prominent nuclear DNA fragmentation and the decreased cardiomyocyte cross-sectional area. This study highlights the potential role of apocynin in inhibiting cisplatin-induced hemodynamic changes, postmitochondrial and mitochondrial damage as indicated by improvement in the state of oxidative stress, inflammation and apoptosis.

1. Introduction

Cisplatin is a potent chemotherapeutic agent with broad spectrum antineoplastic activity against various types of tumors [1]. The optimal effectiveness of cisplatin use is usually limited by several tissues toxicity such as nephrotoxicity and ototoxicity [2,3]. Among the other factors limiting cisplatin use is acute and cumulative cardiovascular complications that can impair the quality of life after treatment. These complications include electrocardiographic changes, arrhythmias, myocarditis, cardiomyopathy, etc. [4]. These cardiac events may lead to cisplatin dose reduction or delay and in some cases, have necessitated the discontinuation of chemotherapy use [5].

Although the mechanisms underlying the antitumor effects of cisplatin are relatively well known, cellular and molecular mechanisms involved in its cardiotoxicity are still not clear [6]. However, several experimental and clinical studies support the view that an increase in oxidative stress is implicated in the cardiotoxicity of cisplatin [1,5,6]. The role of oxidative stress in the pathophysiology of cisplatin-induced toxicity has been shown by using several antioxidants and superoxide dismutase mimetics [7,8]. Oxidative stress favors the transcription and translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) into the nucleus. Activation of Nrf2 stimulates the expression of a great number of genes that are responsible for the synthesis of several antioxidants and heme oxygenase-1 (HO-1) as a signaling pathway to protect the cell against oxidative stress and inflammation [9]. The increased production of reactive oxygen species (ROS) can lead to an enhancement in the expression of nuclear factor kappa B (NF-κB) and the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) which can intensify the cytotoxic effects of cisplatin [10]. Previous studies have also demonstrated the role of mitochondrial damage in several models of cisplatin-induced ototoxicity [11], nephrotoxicity [12] and gastrointestinal toxicity [13]. Mitochondrial dysfunction has been implicated in the cytotoxicity of cisplatin as evidenced by a decline in membrane electrochemical potential [14] and depletion of mitochondrial antioxidant enzymes [15].
NADPH oxidase is an enzyme system that is responsible for significant production of ROS. NADPH oxidase plays an important role in cisplatin-mediated ROS generation and cytotoxicity [16] and therefore inhibition of this enzyme may represent an attractive therapeutic target for the treatment of many diseases. Apocynin, a specific NADPH oxidase inhibitor, is a naturally occurring methoxy-substituted catechol that has been reported to ameliorate tissue damage in several experimental models. Inhibition of NADPH oxidase prevented hemorrhagic shock-induced organ injury [17], hepatic ischemia/reperfusion (I/R) injury [18] and lipopolysaccharide-induced lung injury [19], likely by inhibiting ROS formation via NADPH oxidase. Apocynin has been demonstrated to inhibit not only NADPH oxidase activity but also to prevent the activation of NF-κB, which is an important mediator of inflammation [20]. Due to its high oral bioavailability, low toxicity and high in vivo efficacy, apocynin may serve as a promising therapeutic candidate [21].

Despite the clinical studies, few studies have focused on reducing the cardiotoxicity of cisplatin. Apocynin has previously been used with success to prevent cisplatin-induced nephrotoxicity in rats [22]. Based on these findings, the aim of the present study was directed to investigate the protective effects of apocynin on oxidative stress, inflammation and the subsequent myocardial damage induced by cisplatin cardiotoxicity in rats.

2. Material and methods

2.1. Animals

Male Wistar rats weighing 170–190 g were obtained from the animal facility of the National Institute for Vaccination, Helwan, Egypt. Animals were housed under controlled environmental conditions at constant temperature (25 ± 2 °C) and a 12/12 h light/dark cycle. Rats were acclimatized for 1 week before any experimental procedures and were allowed standard rat chow diet and water ad libitum. The investigation complies with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Ethics Committee for Animal Experimentation at Faculty of Pharmacy, Cairo University.

2.2. Chemicals

Apocynin and cisplatin were obtained from Sigma–Aldrich Chemical Co. (USA).

2.3. Experimental design

Fifty two rats were randomly divided into four groups. Group I (n = 10) served as a normal control group. Group II (n = 10) received apocynin in drinking water for ten successive days. Group III (n = 18) received a single dose of cisplatin (7 mg/kg, ip) on day 6 [1]. Group IV (n = 14) received apocynin in drinking water for five successive days before and after injection of cisplatin. Apocynin was dissolved in drinking water daily at a concentration of 600 mg/L and provided ad libitum to animals [23]. The average volume of apocynin solution consumed by each animal during the treatment period was 25–30 ml/day. Apocynin solution was supplied via cage-side water bottles which were cleaned daily and refilled throughout the whole experimental period. Animals were weighed on day 11. Heart rate (HR) and blood pressure (BP) were measured by the non-invasive tail cuff method using PowerLab data acquisition systems (ADInstruments, Australia). Rats were then anesthetized with thiopental (5 mg/kg, i.p.) and blood was collected from the retro-orbital sinus using non heparinized capillary tubes for serum separation. At the end of the experiment, animals were euthanized and the whole ventricles were rapidly excised, washed with ice-cold saline, dried and weighed. For each group, two sets of experiments were conducted; one for biochemical examination and the other (n = 4) for histological examination.

2.4. Biochemical measurements

The whole ventricles were used where each ventricle was divided into three transverse parts. One part was used for mitochondrial separation. Another part was homogenized in ice-cold saline using a homogenizer (Heidolph Diax 900, Germany) to prepare 5% homogenate. The resultant homogenate was used for determination of TNF-α content as well as NADPH oxidase, myeloperoxidase (MPO) and caspase-3 activities. Finally, the third part was used for estimation of gene expressions and DNA fragmentation.

2.4.1. Isolation of heart mitochondria

Isolation of heart mitochondria was done according to the method of Chappel and Hansford [24]. The part of ventricle used for mitochondrial separation was homogenized in 0.7 M Tris–HCl buffer (pH 7.4) (Sigma–Aldrich Chemical Co., USA) containing 0.25 M sucrose (El-Nasr Pharmaceutical Co., Egypt) and centrifuged at 2500 g for 10 min at 4 °C to remove nuclei and unbroken cells. The supernatant fluid was decanted into eppendorf tubes and centrifuged at 10000 g for 10 min at 4 °C to form primary mitochondrial pellet. The supernatant fluid (postmitochondrial fraction) was removed for further analysis and the pellet was gently resuspended in 0.5 ml Tris–sucrose buffer for washing. The pellet was recentrifuged and the supernatant fluid was decanted. This washing cycle was repeated three times to improve the degree of mitochondrial purity. The final mitochondrial pellet was resuspended in Tris–sucrose buffer. The fresh mitochondrial suspension was used for estimation of mitochondrial membrane potential. The postmitochondrial and mitochondrial fractions were used for assessment of lipid peroxidation products and reduced glutathione as well as the antioxidant enzymes; superoxide dismutase, glutathione peroxidase and catalase activities. The protein contents of tissue homogenate, postmitochondrial and mitochondrial fractions were determined using the method of Lowry et al. [25].

2.4.2. Serum creatine kinase-MB activity

Serum creatine kinase-MB (CK-MB) was assessed using a commercially available kit (Stanbio, USA). Results were estimated kinetically at 340 nm using spectrophotometer (Thermo electron corporation, England) and expressed as U/L.

2.4.3. Lipid peroxidation products

Postmitochondrial and mitochondrial lipid peroxidation products were estimated by determination of the levels of thioarbituric acid reactive substances (TBARS) that were measured according to the assay of Buege and Aust [26] and expressed as nmol/mg protein.

2.4.4. Reduced glutathione

Postmitochondrial and mitochondrial reduced glutathione (GSH) contents were determined using 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) (Sigma–Aldrich Chemical Co., USA), which produces a stable yellow color that can be measured spectrophotometrically at 412 nm [27] and expressed as nmol/100 mg protein.

2.4.5. Superoxide dismutase activity

Postmitochondrial and mitochondrial superoxide dismutase (SOD) activities were assessed according to the method of Marklund [28]. It simply resides on computing the difference between autooxidation of pyrogallol (Sigma–Aldrich Chemical Co., USA)
alone and in presence of the fraction that contains the enzyme. Changes in the absorbance at 420 nm were recorded at 1-min interval for 5 min. Results were expressed as U/mg protein. One unit is defined as the amount of enzyme that produces 1 μmol NADPH per min at 25 °C.

2.4.6. Glutathione peroxidase activity

Postmitochondrial and mitochondrial glutathione peroxidase (GPx) activities were determined according to the method of Paglia and Valentine [29] that depends on oxidation of GSH (Sigma–Aldrich Chemical Co., USA) by hydrogen peroxide (H2O2) (El-Nasr Pharmaceutical Co., Egypt) in the presence of GPx. The decrease in absorbance was measured at 340 nm as NADPH (Sigma–Aldrich Chemical Co., USA) was converted to NADP⁺ reflecting the amount of oxidized glutathione that has been formed and consequently the activity of GPx. Enzyme activity was expressed as U/mg protein, where one unit is defined as the amount of enzyme that oxidizes 1 μmol NADPH per min at 25 °C.

2.4.7. Catalase activity

Postmitochondrial and mitochondrial catalase activities were assessed according to the method of Abei [30]. Catalase reacts with a known quantity of H2O2. The reaction is stopped after exactly one min with sodium azide (Sigma–Aldrich Chemical Co., USA). The remaining H2O2 reacts with 3,5- dichloro-2-hydroxybenzene sulfonic acid and 4-aminoazobenzene (Sigma–Aldrich Chemical Co., USA) to form a chromophore with color intensity inversely proportional to the amount of catalase in the original sample. The color intensity was measured at 510 nm and the results were expressed as U/mg protein. One unit of catalase activity is defined as the amount of enzyme that degrades 1 μmol H2O2 per min at 25 °C.

2.4.8. NADPH oxidase activity

Myocardial NADPH oxidase activity was measured using cytochrome c reductase NADPH assay kit (Sigma, USA). This assay measures the reduction of cytochrome c by NADPH–cytochrome c reductase in the presence of NADPH. The absorption spectrum of cytochrome c changes with its oxidation/reduction state. The reduction of cytochrome c is monitored by the increase in cytochrome c absorbance at 550 nm. Results were expressed as U/mg protein. One unit of enzyme activity will reduce 1 μmol oxidized cytochrome c in the presence of 100 μmol NADPH per minute at pH 7.8 at 25 °C.

2.4.9. Myeloperoxidase activity

Myocardial myeloperoxidase (MPO) activity was determined kinetically at 460 nm by measuring the rate of H2O2-dependent oxidation of o-dianisidine (Sigma–Aldrich Chemical Co., USA) that is catalyzed by MPO [31] and expressed as mU/mg protein. One unit of MPO activity is defined as the amount of enzyme that degrades 1 μmol peroxide per min at 25 °C.

2.4.10. TNF-α

Myocardial TNF-α content was assessed using rat TNF-α ELISA kit (BD Biosciences, San Diego, USA). The procedure of the used kit was performed according to the manufacturer’s instructions and the results were expressed as pg/mg protein.

2.4.11. Caspase-3 activity

Myocardial caspase-3 activity was estimated using caspase-3 colorimetric assay kit (R&D Systems Inc, USA). The absorbance was read at 405 nm using a microplate reader (BioTek instruments, USA). The results were expressed as nmol pNA/h/mg protein.

2.4.12. Mitochondrial membrane potential

Mitochondrial membrane potential was measured in freshly isolated mitochondria using JC-1 assay kit (Sigma, USA) according to the manufacturer’s instructions. The relative fluorescence of the sample was measured at 590 nm after excitation at 490 nm using a spectrofluorophotometer (Shimadzu RF-1501, Japan). Results were expressed as JC-1 transfer rate in mitochondria (fluorescence intensity/min/100 μg protein).

2.4.13. Estimation of Nf-2, HO-1 and NF-kB gene expressions

2.4.13.1. RNA extraction. Total RNA was extracted from heart tissue homogenates using RNeasy Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. RNA concentration and purity were determined spectrophotometrically and its integrity was assessed by gel electrophoresis on 1% agarose gel (Invitrogen Co., USA) stained with ethidium bromide (Sigma-Aldrich Chemical Co., USA).

2.4.13.2. Quantitative real-time PCR technique. In brief, first-strand cDNA synthesis was performed with the SuperScript Choice System (Life Technologies, Breda, Netherlands) according to the manufacturer’s protocol using 1 μg total RNA. For quantitative real-time PCR, 5 μl of first-strand cDNA was used in a total volume of 25 μl, containing 12.5 μl 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 200 ng of each primer. The sequence of the primers used is listed in Table 1. PCR reactions consist of 95 °C for 10 min (1 cycle), 94 °C for 15 s and 60 °C for 1 min (40 cycles). These reactions were performed on an ABI Prism 7900 HT Fast Real Time PCR system (Applied Biosystems). Analysis of relative gene expression was done by evaluating the real-time quantitative PCR data by 2–ΔΔCt method as described previously by Livak and Schmittgen [32] and Pfaffl [33]. β-actin (R&D Systems Inc., USA) was used as a housekeeping gene.

2.4.14. DNA fragmentation

DNA was extracted from heart tissues using Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, USA) according to the manufacturer’s instructions. To detect DNA fragmentation, 10 μg DNA was electrophoretically fractionated on 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. The gel was visualized and photographed under UV light.

2.5. Histological assessment of cardiomyocyte cross-sectional area

For histological examination, the apex of the ventricle was separated, rinsed in ice-cold saline and immediately fixed in 10% formalin for 24 h. Specimens were processed for paraffin embedding, and 5 μm sections were prepared. The sections were stained with haematoxylin and eosin (H&E) (Sigma–Aldrich Chemical Co., USA) and examined microscopically. Images were captured and processed using Adobe Photoshop (version 8.0). Random areas were examined for each group (three sections per animal) and cardiomyocyte cross-sectional area was determined. For each section, 150 cardiac muscle cells were measured in μm² and an average value was calculated.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
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<tr>
<td>Nrf2</td>
<td>F: 5’-CAAGTGGCAGTTCATATTAC-3’; 5’CAGAAATGTTGTCGCTG-3’</td>
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<tr>
<td>HO-1</td>
<td>F: 5’-GGCTCTGACTCTGTCTC-3’; 5’-GGCACTTCCCTTTAGCC-3’</td>
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<tr>
<td>NF-kB</td>
<td>F: 5’-CCGCTGCCAACTCTCAATC-3’; 5’-GAGACCCGCTGCC-3’</td>
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<tr>
<td>β-actin</td>
<td>F: 5’-TCTGCCACACACTCTACATAC-3’; 5’-AGCCACAACCCCTG-3’</td>
</tr>
<tr>
<td>TGG ACA CT-3</td>
<td>F: 5’-GCCATCCAGACGCACACA-3’; 5’-GGCCAAAGCTGCAT-3’</td>
</tr>
</tbody>
</table>

Table 1

The sequence of the primers used for real-time -PCR analysis.
2.6. Statistical analysis

All data obtained were presented as means±S.E.M. Results were analyzed using one way analysis of variance test (One-way ANOVA) followed by Student–Newman–Keuls multiple comparison test. Statistical analysis was performed using GraphPad Instat software (version 2.04). For all the statistical tests, the level of significance was fixed at p<0.05.

3.1. Results

3.1. Body weight and percentage of mortality

Cisplatin-treated group showed a significant decrease in body weight (175.33 ± 3.62 vs. 206.14 ± 3.55 g) and 39% mortality. Pretreatment with apocynin pronouncedly alleviated the decrease in body weight and significantly reduced the percentage of mortality to 14% (Table 2).

3.2. Heart rate and mean arterial blood pressure measurements

The current study revealed that administration of a single dose of cisplatin (7 mg/kg) resulted in a significant decrease in both heart rate (294.00 ± 6.57 vs. 340.67 ± 8.82 beats/min) and mean arterial blood pressure measurements (94.33 ± 2.79 vs. 121.29 ± 2.62 mm Hg). Pretreatment with apocynin restored the heart rate and blood pressure to their basal normal values (Table 2).

3.3. Biochemical, molecular and histological measurements

The present data show a significant elevation of serum CK-MB activity in cisplatin-treated group where apocynin pretreatment induced a significant reduction of serum CK-MB activity in rats exposed to cisplatin treatment (Table 2).

The present results clearly indicate significant alterations in the biochemical markers of oxidative stress in both postmitochondrial and mitochondrial fractions in cardiac tissues of cisplatin-treated rats. This was demonstrated by a significant elevation of TBARS (Fig. 1A) and a significant reduction of reduced glutathione content (Fig. 1B), SOD (Fig 2A), GPx (Fig. 2B) and catalase (Fig. 2C) activities in both postmitochondrial and mitochondrial fractions. Pretreatment with apocynin succeeded to alleviate the previously mentioned changes induced by the state of oxidative stress (Figs. 1 and 2). The enhanced state of oxidative stress in cisplatin-treated group was associated with a significant elevation of NADPH oxidase activity (Fig. 3) which has been reported to be the major source of ROS production. On the other hand, apocynin significantly alleviated the elevation of NADPH oxidase activity. This was associated with an improvement in the state of oxidative stress as observed in the present study.

Moreover, the predominance of oxidative stress status in cisplatin-treated group was supported by twofold increase in Nrf2 (Fig. 4A) and sixfold increase in HO-1 (Fig. 4B) expressions. Both expressions were significantly downregulated in apocynin pretreated group indicating an amelioration of the state of oxidative stress and an alleviation of the compensatory increase in both of Nrf2 and HO-1 expressions.

Furthermore, oxidative stress elicited the activation of the expression of redox-sensitive transcription factor; NF-κB (Fig. 4C) in cisplatin-treated group. This was associated with a significant elevation of both TNF-α content and MPO activity (Fig. 5). In the present study, apocynin significantly decreased these markers of inflammation.

Our results demonstrated that mitochondria in rat heart treated with cisplatin remarkably showed a loss of mitochondrial transmembrane potential indicating an early event in apoptosis (Fig. 6A). This was confirmed by significant increase in myocardial caspase-3 activity (Fig. 6B) and significant decrease in cardiomyocyte cross-sectional area (Fig. 7). Moreover, cisplatin treatment resulted in a marked decrease in the amount of intact genomic DNA in cardiac tissues, along with degradation of DNA, characterized by mixed smearing and laddering, giving an indication of apoptotic cell death (Fig. 6C). Pretreatment with apocynin revealed preserva-

Table 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mortality %</th>
<th>BW (g)</th>
<th>HR (beats/min)</th>
<th>MAP (mm Hg)</th>
<th>Serum CK-MB (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
<td>206.14 ± 3.55</td>
<td>340.67 ± 8.82</td>
<td>121.29 ± 2.62</td>
<td>57.36 ± 4.17</td>
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<tr>
<td>Apocynin</td>
<td>0</td>
<td>205.57 ± 4.23</td>
<td>343.67 ± 7.55</td>
<td>119.00 ± 2.18</td>
<td>61.70 ± 7.3</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>39</td>
<td>175.33 ± 3.62</td>
<td>294.00 ± 6.57</td>
<td>94.33 ± 2.79</td>
<td>104.57 ± 12.3</td>
</tr>
<tr>
<td>Cisplatin + apocynin</td>
<td>14</td>
<td>200.83 ± 8.13</td>
<td>322.17 ± 7.13</td>
<td>115.33 ± 4.73</td>
<td>83.12 ± 5.23</td>
</tr>
</tbody>
</table>

Each value represents the mean of 6–8 experiments ± S.E.M. 
*p<0.05 vs. normal.
*#p<0.05 vs. cisplatin.

Fig. 1. Effect of apocynin on cisplatin-induced changes in oxidative stress markers in postmitochondrial and mitochondrial fractions in heart tissues of rats. (A) Thiobarbituric acid reactive substances (TBARS). (B) Reduced glutathione (GSH). Each value represents the mean of 6–8 experiments ± S.E.M. *p<0.05 vs. normal, *#p<0.05 vs. cisplatin.
tion of both mitochondrial transmembrane potential and intact genomic DNA in addition to normalization of caspase-3 activity and preservation of cardiomyocyte cross-sectional area.

4. Discussion

In the present study, the protective effects of apocynin were investigated on oxidative stress, inflammation and the subsequent myocardial damage induced by cisplatin cardiotoxicity in rats. The present data show cardiac dysfunction which was manifested by the reduction of both BP and HR. Cisplatin-induced cardiotoxicity was reported to be due to its disposition in the sinoatrial-node area, which may lead to bradycardia [4]. Moreover, left ventricular dysfunction and depressed cardiomyocyte contraction could contribute to HR and BP reduction [6]. These hemodynamic abnormalities could be related to mitochondrial abnormalities, enhanced oxidative stress and apoptosis as indicated in the present investigation. Prophylactic treatment with apocynin efficiently ameliorated the decrease in both HR and BP. Apocynin was previously reported to improve diastolic dysfunction in angiotensin II-induced hypertensive mice via ameliorating oxidative stress and cardiac fibrosis [34].

Cisplatin increased serum CK-MB activity in the present investigation. This effect could be a secondary event to increased lipid peroxidation of cardiac membranes as indicated in the present study. This could result in irreversible modification and damage of membrane structures with consequent leakage of cardiac enzymes [35]. Pretreatment with apocynin significantly decreased the serum CK-MB level compared to cisplatin group. The improved antioxidant status by apocynin pretreatment in the present

Fig. 2. Effect of apocynin on cisplatin-induced changes in antioxidant enzymes in postmitochondrial and mitochondrial fractions in heart tissues of rats. (A) Superoxide dismutase (SOD). (B) Glutathione peroxidase (GPx). (C) Catalase. Each value represents the mean of 6–8 experiments ± S.E.M. *p<0.05 vs. normal, #p<0.05 vs. cisplatin.

Fig. 3. Effect of apocynin on cisplatin-induced changes in NADPH oxidase activity in heart tissues of rats. Each value represents the mean of 6–8 experiments ± S.E.M. *p<0.05 vs. normal, #p<0.05 vs. cisplatin.

Fig. 4. Effect of apocynin on cisplatin-induced changes in gene expressions in heart tissues of rats. (A) Nuclear factor erythroid 2-related factor 2 (Nrf2). (B) Heme oxygenase-1 (HO-1). (C) Nuclear factor kappa B (NF-κB). Each value represents the mean of 6–8 experiments ± S.E.M. *p<0.05 vs. normal, #p<0.05 vs. cisplatin.
Oxidative stress and inflammation are among the most critical factors implicated in cisplatin-induced toxicity. Results of the current study revealed a state of oxidative stress in postmitochondrial and mitochondrial fractions of heart tissues as indicated by a significant increase in TBARS and a significant decrease in GSH together with a notable inhibition of the antioxidant enzymes activities such as SOD, GPx and catalase. The heart is an organ that is susceptible to oxidative stress since cardiac tissue has very low level of antioxidant enzymes such as catalase and SOD. Increased oxidative stress causes damage to cellular macromolecules such as nucleic acids, proteins and lipids. The depletion of GSH is an early and a critical event during cisplatin-induced lipid peroxidation and subsequent toxicity. The formation of the platinum-GSH conjugate may account for GSH depletion and eventually for the alteration of the redox state. The reduction of GSH may be also explained by the decreased activity of glutathione reductase resulting from a direct attack of cisplatin. Moreover, the decreased SOD activity in the heart tissue of cisplatin-treated rats has been reported to be due to the loss of copper and zinc which are essential for the enzyme activity. Pretreatment with apocynin succeeded to normalize myocardial TBARS content and effectively restored the altered antioxidant enzymes activities. Interestingly, apocynin administration to normal or cisplatin-treated rats significantly increased myocardial GSH content above normal value. Apocynin has previously been demonstrated to enhance the activity of antioxidant factors and protect the cellular membranes against lipid peroxidation implicated in the pathogenesis of diabetic neuropathy. This probably occurs due to the inhibitory influence of apocynin on NADPH oxidase.

In the present investigation, cisplatin-treated group showed a significant elevation of NADPH oxidase activity. The enhanced activity of NADPH oxidase has been associated with oxidative stress in cisplatin-treated group. NADPH oxidase has been reported to play a critically essential role in cisplatin-mediated ROS generation and ototoxicity. NADPH oxidase which is a major source of endoplasmic reticulum stress could initiate oxidative stress at early stages of cardiotoxicity and then trigger itself and other ROS sources leading to the progression of oxidative stress. NADPH oxidase together with other enzymes, e.g. xanthine oxidase and lipoxygenases appear to act synergistically to augment ROS generation. The present data show an inhibition of NADPH oxidase activity in apocynin pretreated animals. Apocynin, being a specific NADPH oxidase inhibitor, has the ability to inhibit not only the activity of NADPH oxidase but also the expression of this enzyme. Apocynin probably inhibits NADPH oxidase through preventing the assembly of its multi-subunits by reacting with thiol groups essential for enzyme assembly.

In a previous study, apocynin in drinking water has been found to reduce the overproduction of superoxide and protein oxidation products induced by angiotensin II in left ventricle, likely via inhibition of NADPH oxidase.

**Fig. 5.** Effect of apocynin on cisplatin-induced changes in inflammatory markers in heart tissues of rats. (A) tumor necrosis factor-α (TNF-α). (B) Myeloperoxidase (MPO) activity. Each value represents the mean of 6–8 experiments ± S.E.M. *p<0.05 vs. normal, #p<0.05 vs. cisplatin.

**Fig. 6.** Effect of apocynin on cisplatin-induced changes in apoptotic markers in heart tissues of rats. (A) Mitochondrial membrane potential (JC-1 transfer rate in mitochondria). (B) Caspase-3 activity. (C) DNA fragmentation using agarose gel electrophoresis of DNA isolated from heart homogenate. M: DNA marker with 100 bp. Each value represents the mean of 6–8 experiments ± S.E.M. *p<0.05 vs. normal, #p<0.05 vs. cisplatin.
The Nrf2/HO-1 signaling pathway plays an important role as a compensatory mechanism in response to cell exposure to oxidative stress and inflammation. Upon exposure to oxidative stress, Nrf2 translocates into the nucleus with upregulation of a number of antioxidant genes in response to a wide array of stimuli to protect the cell against oxidative stress and inflammation [46]. This has been correlated with decreased levels of catalase, GPx and SOD indicating the predominance of oxidative stress side. Apocynin prevented the adaptive induction of Nrf2-mediated HO-1/NF-κB expressions in cisplatin-treated group via the attenuation of oxidative stress as revealed in the present study. Consistent with our results, Lee et al. [47] recently reported that ginsenoside through its ability to scavenge ROS, reduced Nrf2-mediated HO-1 expression in cisplatin-induced nephrotoxicity and hepatotoxicity.

Furthermore, oxidative stress induced the expression of redox-sensitive transcription factor (NF-κB) in cisplatin-treated group along with a significant elevation of both TNF-α content and MPO activity. Cisplatin-mediated elevation of pro-inflammatory cytokines is an upstream signal of ROS production [16]. NF-κB plays a central and a crucial role in inducing the expression of inflammatory cytokines such as TNF-α [48]. TNF-α is a potent trigger that is involved in inflammatory cells infiltration as supported by the significant increase in MPO activity in the present study. Translocation of Nrf2 into nucleus has previously been demonstrated to enhance the upregulation of NF-κB and pro-inflammatory cytokines after traumatic brain injury [49,50]. In the present study, apocynin significantly decreased the markers of inflammation such as TNFα and MPO as well as NF-κB expression. This is in agreement with earlier reports demonstrating the anti-inflammatory and the antioxidant effects of apocynin. Apocynin inhibits NADPH oxidase which is the major pathway for modulation of ROS production by MPO in activated neutrophils [51]. Apocynin has been shown to attenuate TNF-α and IL-1 production in splanchnic artery occlusion and reperfusion in rats ileum most likely via inhibition of the activation of NF-κB [52,53]. Moreover, apocynin effectively attenuated lung I/R injury by inhibiting inflammatory responses associated with the generation of ROS. Apocynin proved to be a potent anti-inflammatory agent, based on the selective inhibition of superoxide anion production from activated neutrophils and thus the inhibition of NF-κB and the pro-apoptotic pathway activation [54].

Administration of cisplatin significantly disrupted the integrity of the electrochemical gradient within mitochondria in the present

Fig. 7. Representative images revealing cardiomyocyte cross-sectional areas in normal (A), apocynin (B), cisplatin (C) and cisplatin + apocynin (D) groups (H&E ×200). (E) cardiomyocyte cross sectional area in μm². Each value represents the mean of four experiments ± S.E.M. *p<0.05 vs. normal, #p<0.05 vs. cisplatin.
study. Being highly enriched with mitochondria, cardiac myocytes accumulate a large amount of cisplatin and exhibit enhanced mitochondrial injury [55]. In the present study, a significant elevation of caspase-3 activity, a dramatic nuclear DNA fragmentation and decreased cardiomyocyte cross-sectional area were observed in the heart tissues after cisplatin treatment, possibly indicating apoptotic cell death that contributed to these results [56,57]. Furthermore, the disruption of the mitochondrial transmembrane potential by cisplatin may indicate an impairment of mitochondrial function that precedes initiation of apoptosis. This is closely related to the occurrence of oxidative stress, as evidenced by mitochondrial oxidative damage and redox state alteration. A central role of mitochondrial dysfunction has previously been suggested in the mechanism underlying cisplatin-induced nephrotoxicity [39]. Oxidative stress and mitochondrial oxidative damage have been implicated in the etiology of toxicity resulting from exposure to platinum compounds. Mitochondrial membrane potential is a good indicator of the energy status of mitochondria in particular and of cellular homeostasis in general [58]. Pretreatment with apocynin revealed a significant preservation of mitochondrial transmembrane potential compared to cisplatin-treated group. Choi and Lee [58] demonstrated a significant role of apocynin in inhibition of antymycin A-induced toxicity and cell damage in osteoblastic MC3T3-E1 cells by preventing mitochondrial membrane potential dissipation. This was attributed to its antioxidant effects and the attenuation of mitochondrial dysfunction. Pretreatment with apocynin revealed also a significant decrease in caspase-3 activity and nuclear DNA fragmentation together with preservation of cardiomyocyte cross-sectional area compared to cisplatin-treated group. A potential mechanism for this therapeutic effectiveness could be related to the ability of apocynin to attenuate the severity of the cellular stress that favors activation of apoptotic pathways.

In conclusion, the main mechanisms of cisplatin-induced cardiotoxicity might be related to enhancement of oxidative stress and inflammatory pathways. These situations could disrupt the integrity of mitochondrial membrane in addition to elevated caspase-3 activity and DNA fragmentation. Pretreatment with apocynin was effective in ameliorating cisplatin-induced hemodynamic changes and in alleviating the state of oxidative stress, inflammation and apoptosis. The effectiveness of apocynin in restoring the mitochondrial function or inhibiting oxidative stress in the heart may be a promising way to attenuate cisplatin-induced cardiotoxicity. Finally, clinical studies are required to establish the beneficial effectiveness of apocynin as a possible approach to reduce acute and cumulative cardiovascular complications resulting from the use of cisplatin.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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