Effects of resveratrol on carbon monoxide-induced cardiotoxicity in rats

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ABSTRACT

Carbon monoxide (CO) poisoning leads to tissue hypoxia resulting in cardiovascular disturbances. Resveratrol (RES) is considered a natural cardioprotective agent especially in the setting of ischemia/reperfusion injury. In the present study, the cardioprotective potential of RES against CO-induced cardiotoxicity was evaluated. 45 male Wistar rats, animals were randomly assigned to 5 experimental groups. The first group served as negative control and was not exposed to CO. All remaining rats were exposed to CO 3000 ppm for 60 min. The second group received normal saline following CO exposure, while groups 3, 4 and 5 were injected intraperitoneally with different doses of RES (1, 5 and 10 mg/kg, respectively). Histopathological examination showed that RES administration reduced myocardial lesions compared to control groups. Myocardial Akt expression was significantly increased in rats treated with the highest dose of RES (p < 0.05) compared to CO-exposed normal animals. Caspase-3 activity in rat cardiomyocytes of RES-treated animals was significantly decreased in a dose-dependent manner. ECG findings did not differ significantly among CO-exposed groups. In conclusion, the present study offers evidence of a protective effect of RES administration on CO-induced cardiotoxicity via Akt up-regulation and attenuation of caspase-3 activity in rat hearts.

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1. Introduction

Carbon monoxide (CO) is a colorless, odorless, non-irritating gas that is mainly produced by incomplete combustion of fossil fuels. CO poisoning represents a significant public health problem. In the USA, annually near 20,000 emergency department visits are attributed to CO poisoning (\textit{Control and Prevention, 2007}). In Iran annually CO poisoning accounts for 3.1–11.6% of all poisoning incidents with a reported 20% fatal outcome (Sheikhzadeh et al., 2010). CO poisoning primarily affects the brain and the heart which are the organs that have greater oxygen demands. The heart is affected early after severe CO poisoning and the main cardiotoxic effects of CO are myocardial infarction (MI), cardiomyopathy, cardiac arrhythmias, hypotension, cardiac arrest and death (Procop and Chichkova, 2007). Inhibition of oxygen exchange at cellular level, oxidative stress, and cardiomyocyte apoptosis and necrosis are the most important mechanisms that are involved in CO-induced cardiotoxicity, with hypoxia at the cellular level probably playing the key role (Ernst and Zibrok, 1998; Satran et al., 2005).

"Nutraceuticals" are nutritional agents with pharmacological properties. Resveratrol (RES) (trans-3,4,5-trihydroxystilbene) is a nutraceutical polyphenolic phytoalexin compound that is found in grape skin and red wine and is reported to contribute to the cardiovascular protective effects of red wine (Fu, 2015). The pharmacological properties of RES expand to anti-cancer, antioxidant, neuroprotective, anti-inflammatory and cardioprotective effects (Androutsopoulos et al., 2011; Pangeni et al., 2014). RES
is considered to be cardioprotective especially in the setting of ischemia/reperfusion injury.

RES cardioprotection comes as a result of its effect on reducing infarct size, inducing autophagy, preventing heart failure and activating cell survival proteins including Bcl-2 and protein kinase B (Akt) (Chen et al., 2009; Gurusamy et al., 2009). The serine-threonine kinase Akt is a pro-survival protein that is considered as a valuable target for the evaluation of the protective effects of RES. Recent studies have demonstrated both in vitro and in vivo that RES, through upregulation of sirtuins, a group of enzymes with deacetylation activity, can stimulate the IGF-1/Akt pro-survival signaling, enhance oxidative stress defense and inhibit the mitochondrial membrane permeabilization pores from opening thus leading to the prevention of senescence of myocardial cells (Cappetta et al., 2016). At the same time, RES possesses significant anti-arrhythmic properties, as it is capable of increasing the cardiac effective refractory period mainly through inhibiting ionic channels including L(Na), L(to) and L(ss), suppressing the possibility of induction of serious arrhythmias especially in the setting of ischemia/reperfusion injury (Chen et al., 2007).

In recent studies, beneficial effects of erythropoietin on CO-induced cardiotoxicity were shown and it became evident that one of the key mechanisms involved in myocardial salvage was attenuation of myocardial apoptosis (Rezaee et al., 2016; Shahsavad et al., 2012). Apoptosis which is a mechanism for eliminating redundant cells is also a key factor in the pathogenesis of heart diseases like heart failure. Induction of apoptosis is associated with activation of aspartate-specific cysteine proteases like caspase-3. In this context, mitochondria play an important role in apoptosis by releasing cytochrome c and activating caspase-9, which activates caspase-3, the molecule responsible for DNA cleavage (Liao et al., 2015).

The present study evaluated the cardioprotective effects of RES in a setting of high CO exposure. Keeping in mind that in CO poisoning the ultimate effect is the induction of hypoxia at the cellular level, a mechanism that resembles cardiac ischemia, we hypothesized that RES may ameliorate the deleterious effects of CO poisoning in rats’ hearts. For this purpose, male Wistar rats were exposed to high CO concentration and subsequently received different doses of RES. Cardiac histopathology, ECG recordings, measurement of Akt and caspase-3 levels were performed to assess a possible cardioprotective effect of RES against CO poisoning and its effect on apoptosis.

2. Material and methods

2.1. Animals

Forty five male Wistar rats (8–10 weeks age; 200–250 g), obtained from the animal house of Zabol University of Medical Sciences, Zabol, Iran, were kept under standard conditions (free access to food and water, temperature 25 °C, 12 h/12 h light/dark cycles). All animals were treated in accordance with the guidelines for care and use of laboratory animals prepared by the Animal Research Ethic Committee of Zabol University of Medical Sciences, Zabol, Iran and in conformity with EU Directive 2010/63/EU for animal experiments. The study was approved by the Animal Research Ethic Committee of Zabol University of Medical Sciences.

Animals were randomly assigned to 5 experimental groups. The first group served as negative control and was not exposed to CO. All remaining rats were exposed to CO 3000 ppm for 60 min. The second group received normal saline following CO exposure, while groups 3, 4 and 5 were injected intraperitoneally with different doses of RES (1, 5 and 10 mg/kg, respectively) in order to achieve faster systemic bioavailability (Wang, 2013 #65).

2.2. Chemicals

Akt protein and anti-β Actin and secondary rabbit antibody were purchased from Cell Signaling (Beverly, MA, USA). Coomassie (Bradford) Protein Assay Kit was purchased from Thermo scientific (Rockford, USA). Carbon monoxide capsule (99.999% purity) was obtained from Darman Gas (Tehran, Iran). Power Lab (AD Instruments, Bella Vista, New South Wales, Australia) was used to record ECGs. Caspase-3/CPP32 Colorimetric Assay Kit was obtained from Bio Vision (USA). Pierce® BCA Protein Assay Kit was purchased from Thermo Scientific (USA) and RES was obtained from Sigma-Aldrich Chemical Co.

2.3. Experimental groups and study design

The animals were placed in a 12 L airtight Plexiglas container with entrance and exit taps. The Plexiglas apparatus was connected via polyethylene glycol (PEG) tubes to CO and oxygen sources. CO was transferred to the container at a constant flow. CO concentration in the 12L container was continuously monitored by a CO analyzer (TP1707 Carbon Monoxide Analyzer, Korea). Animals that were placed in the Plexiglas container were exposed to CO 3000 ppm for 60 min (Ghorbani et al., 2015; Moallem et al., 2015). After CO exposure, animals were removed from the box and received RES 1, 5 and 10 mg/kg/day intraperitoneally (i.p.) for five consecutive days according to the experimental scheme. On day 5, all animals were anesthetized by ketamine (100 mg/kg) and xylazine (10 mg/kg) and sacrificed. After opening the chest cavity, hearts were excised immediately. Heart tissue samples were originally fixed in formalin 10% for 24 h before hematoxylin/eosin staining. The remaining of the hearts were rinsed in saline solution, cut into pieces and samples of about 200 mg taken from the left ventricle of each heart were homogenized (1000 rpm for 10 min) in buffer solution [50 mmol/l Tris-HCl pH 7.5/150 mmol/l NaCl/1% SDS/protease inhibitor cocktail (Sigma-Aldrich)]. Protein content in the supernatants was measured by Bradford protein assay kit. After dilution, protein levels of all samples were identical. Finally, the samples were either used freshly or stored at −70 °C.

In order to determine Akt protein content of the left ventricle, 5–10 μL of the supernatants was loaded on SDS page wells and proteins were separated using gel electrophoresis. At the end of electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) using transfer buffer (25 mMTris, 1.2 mM glycin, 20%methanol, pH 8.0). The membrane was washed three times; each time for 5 min, with tris-buffered saline (TBS). The blot was incubated with enzyme conjugate containing 10% blocking solution on rocker at room temperature for 1 h. In order to remove any unbound conjugate proteins, the membrane was washed three times, each time 5 min, in washing buffer (Hashemzaei et al., 2016). Then, samples were treated with secondary antibody, washed thoroughly with TBS and TWEEN 20 (TBST) and visualized by means of enhanced chemiluminescence 500–1000 μL (Pierce, USA). Finally, the amounts of proteins were calculated using Image j software (USA) following imaging in the dark room.

2.4. Carboxyhemoglobin level assessment

Immediately after CO exposure and before RES administration, blood samples were taken from the rat tail vein from all group animals and heparinized. Carboxyhemoglobin levels were assessed by spectrophotometer calibrated for rat blood (Jenway 6305; Bibby Scientific Ltd., Staffordshire, UK) (Rodkey et al., 1979). Carboxyhemoglobin levels were assessed in all groups in order to confirm the induction of CO toxicity.
2.5. Caspase-3 activity in rats heart

Caspase-3 activity was measured by Caspase-3/CPP32 Colorimetric Assay Kit (BioVision, Palo Alto, CA) (Iwai-Kanai et al., 1999). In brief, the heart tissues were homogenized and centrifuged at 10,000 rpm for 1 min at 4°C. Then, the supernatants were extracted and assayed for caspase-3 activity. Samples (100 μg) of the extracted protein (supernatants) were incubated with the reaction buffer and Ac-DEVD-p-nitroanilide (pNA) for 1 h at 37°C. Enzyme-catalyzed release of pNA was measured at 405 nm using spectrophotometer. For measurement of protein levels in the supernatant, Bradford protein assay kit was used. The total protein was normalized (all supernatant was identical in protein content) and caspase-3 activity was measured using the following equation:

\[
\text{% control activity} = \frac{\text{sample fluorescence}}{\text{total sample lysate protein}} \times \frac{\text{mean of media concentration}}{\text{mean of total media control lysate protein}} \times 100
\]

2.6. ECG recording

ECG in lead II was recorded using PowerLab (ADInstruments, Bella Vista, New South Wales, Australia) before, during, and 2, 3, and 4h after CO poisoning. Heart rate was recorded and ECG was analyzed with respect to ST segment and T wave changes and the presence of atrioventricular (AV) block type 1 and 2, Q waves, sick sinus syndrome, atrial fibrillation, premature ventricular beats and ventricular tachycardia and fibrillation.

2.7. Histopathological examination

From the original 45 male Wistar rats, 30 animals were examined histopathologically (6 animals from each experimental group) by a blind pathologist. After fixing the hearts in the formalin 10% for at least 24h, slices with 4–5 μm thickness were prepared for hematoxylin/eosin staining and abnormalities (i.e necrotic foci and lymphatic infiltration) were assessed (Louzada et al., 2010). Pathological findings were categorized in three grades based on the severity of insults (Mohamadpour et al., 2012). Grade one represented dispersed necrotic cells and/or lymphatic infiltration (Fig. 1B), grade 2 represented necrotic unifocal and/or bifocal area (Fig. 1A), and grade 3 was defined as the presence of more than two necrotic areas (Fig. 1C).

2.8. Statistical analysis

Data were analyzed using SPSS version 16 (SPSS, Inc, Chicago, Illinois, USA). Two-way ANOVA was used to compare continuous variables and Chi-square and Fisher’s exact test for categorical variables. A p-value of <0.05 was considered as statistically significant.

3. Results

3.1. Carboxyhemoglobin concentration after exposure to CO 3000 ppm

The mean blood concentration of carboxyhemoglobin of CO-exposed rats was 70 ± 8 ppm.

3.2. Effect of RES on ECG changes after exposure to CO 3000 ppm

No significant ECG changes were observed in animals exposed to CO and treated with RES in comparison with the CO-exposed saline-treated control group.

3.3. Effect of RES on histological findings after exposure to CO 3000 ppm

Histopathological examination revealed that RES reduced cardiomyocyte necrosis following CO poisoning (Table 1).

3.4. Effect of RES on Akt protein levels following exposure to CO 3000 ppm

RES (10 mg/kg) significantly increased Akt protein expression following CO poisoning compared to saline control group. RES effects on Akt protein expression were dose-dependent as lower doses of RES (1 and 5 mg/kg) showed no marked effect on Akt expression in comparison with saline-treated control group (Fig. 2).

3.5. Effect of RES on caspase-3 activity in rats cardiomyocytes following exposure to CO 3000 ppm

RES significantly decreased caspase-3 activity following CO poisoning in a dose-dependent manner, R² = 0.9711 (Fig. 3).

4. Discussion

RES is a naturally occurring compound, abundant in the skin of black grapes, which possesses significant protective properties especially in the setting of augmented cardiomyocyte stress. Hwang et al. showed that RES alleviates cardiac cell injury caused by H₂O₂-induced stress and suggested that this effect is mediated by the activation of AMP-kinase cascade (Iwai-Kanai et al., 1999). Cardioprotective effects of RES were also reported by Lin et al. who showed that following ischemia/reperfusion, administration of RES (10 mg/kg) for four weeks, decreased the infarct size and ameliorated LV systolic and diastolic function (Lin et al., 2008). On the other hand, RES, as a natural polyphenol, seems to have the ability to stimulate mitochondrial metabolism, up-regulate the overall

Fig. 1. Histological findings following CO 3000 ppm intoxication. (A) Grade 2 pathological necrosis: necrotic unifocal and/or bifocal area. (B) Dispersed necrotic cells and/or lymphatic infiltration. (C) More bifocal necrotic areas (40×).
expression of the components of respiratory chain and enhance oxygen uptake (Lopes Costa et al., 2014). In the present study, administration of different and relatively low doses of RES resulted in attenuation of histopathological lesions in rat’s heart tissue following CO intoxication.

So far, there has been only limited data on the role of apoptosis in CO-induced cardiac injury. A recent study revealed the presence of myocardial apoptosis following CO intoxication in rats and showed the beneficial role of erythropoietin administration in apoptosis suppression and myocardial salvage (Rezaee et al., 2016). In addition, in the present study, RES administration resulted in reduced cardiomyocytes apoptosis through Akt protein up-regulation, which is one of the main pathophysiological ways of apoptosis down-regulation. Akt and the anti-apoptotic protein Bcl-2 survival pathway activation play a pivotal role in attenuation of cardiomyocytes death following ischemia/reperfusion injury (Nitulescu et al., 2016). Moreover, Lekli et al. suggested that other mechanisms induced by the activation of Akt-Bcl-2 survival pathway, were implicated in the increased cardioprotection offered by 1-month pretreatment with RES before ischemia/reperfusion (Lekli et al., 2010).

PI3/Akt pathway is a key signal transduction system of cell survival in cardiomyocytes. Following activation of PI3/Akt, transcription of pro-apoptotic proteins including Bax and caspase-3 decreases and leads to a reduction of myocardial apoptosis (Cardone et al., 1998). Up-regulation of Akt transcription inhibits conformational changes in the pro-apoptotic Bax protein and its translocation to mitochondria, thus prevents disruption of mitochondrial inner membrane and activation of caspase-3 (Yamaguchi et al., 2001 Yamaguchi and Wang, 2001). It is well known that an increase in Bax protein levels or suppression of the pro-survival Bcl protein is a cornerstone in the activation of the intrinsic apoptotic pathway (mitochondrial pathway) (Yamaguchi et al., 2001 Yamaguchi and Wang, 2001). Caspase-3, a common component of apoptotic signaling, mediates both mitochondria-dependent and death receptor-dependent apoptotic pathways (Shahsavand et al., 2012). In the present study, RES administration significantly ameliorated caspase-3 levels indicating a favorable effect of RES in myocardial apoptotic cell death. It is interesting, though, that the lowest dose of RES (1 mg/kg) succeeded in significantly decreasing caspase-3 levels, while its effect on Akt was not equally significant.

On the other hand, RES can induce Akt up-regulation and increase myocardial cell survival through an additional mechanism called autophagy in the setting of ischemia/reperfusion injury (Gurusamy et al., 2009). Autophagy is a catabolic process, through which the cells’ individual components such as damaged
or long-lived proteins and macromolecules, are degraded using the lysosomal machineries. Under normal conditions, autophagy occurs at low levels. Low doses of RES (2 and 5 mg/kg/day) are found to relatively induce autophagy and offer cardioprotection in ischemia/reperfusion, while higher doses (100 mg/kg/day) attenuate autophagy and abolish cardioprotection (Gurusamy et al., 2008). This cardioprotection is the result of a complex interplay between Akt, its upstream mammalian target of rapamycin (mTOR) protein and AMP-activated protein kinase (AMPK). It seems that up-regulation and activation of Akt by low doses of RES employ different and potentially not contradicting mechanisms of cardioprotection, a concept that could explain the favorable effects of RES in CO-induced cardiotoxicity observed in the present study.

One of the most important consequences of CO-induced cardiotoxicity is the occurrence of arrhythmias, especially ventricular arrhythmias mainly as a result of myocardial tissue hypoxia. ECG alterations following CO poisoning include ST-segment (depression and elevation) and T-wave changes, heart block and ventricular arrhythmias. In the present study, no significant effect of RES on ECG parameters was observed in comparison with the control group. In a recent study, it was also reported that RES pretreatment had no effect either on ischemia-induced arrhythmias or on mortality, but it reduced arrhythmias after ischemia/reperfusion (Hung et al., 2000). Liman et al. (2000) confirmed the anti-arrhythmic effects of RES in animal models. However, in both studies, animals were pre-treated with RES while in the present study, RES was administered following CO poisoning. RES administration after CO exposure and its favorable effects on rats’ heart, as observed in the present study, despite any significant anti-arrhythmic effect, could have valuable pathophysiological and therapeutic implications.

CO poisoning usually is diagnosed clinically, evidence of recent CO exposure is needed along with relevant symptoms and demonstration of elevated carboxyhemoglobin levels (Hampson et al., 2012). In the present study, carboxyhemoglobin levels, the major indicator of CO poisoning, reached 70 ppm. Our findings are consistent with previous reports and findings that showed that 60-min CO exposure at the concentration of 3000 ppm results in carboxyhemoglobin levels in the range of 60–76% (Ghorbani et al., 2015; Hashemzaei et al., 2016; Mohamadpour et al., 2012). In the current study there are, however, a few limitations. Lack of direct measurement of autophagy and the lack of functional assessment of severity of myocardial injury by echocardiography following CO poisoning and RES administration are a limitation of the study. Secondly the lack of measurements of myocardial injury as troponin I consist a limitation.

5. Conclusion

Low doses of RES were capable of attenuating the apoptotic signal induced by CO exposure in rat’s heart, evident by amelioration of caspase-3 levels in RES-treated animals. Key player in the mentioned favorable effect was Akt up-regulation. Further research is needed to fully elucidate the cardioprotective potential of RES and the complex interplay of the involved molecules in signal transduction.

Declaration of interest

There is no conflict of interest.

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