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Abstract
Carbon monoxide (CO) poisoning causes cardiotoxicity and so far, no definite antidote has been proposed to overcome CO-induced adverse outcomes. Hesperidin, a citrus flavonoid, has shown cardioprotective effects in cardiac ischemia/reperfusion models. This study investigated the protective effects of hesperidin against CO-induced cardiac injury. To induce CO poisoning, rats were exposed to CO at 3000 ppm for 60 min. On the exposure day and the four following days, hesperidin (at three different doses of 25, 50, and 100 mg/kg/day) was administered intraperitoneally. A group of animals received normal saline and served as the control group. The electrocardiogram (ECG) was recorded and evaluated with special focus on S–T segment changes (depression or elevation), T-wave alterations, AV block and ventricular and supraventricular arrhythmias. On day 6 (i.e., the day after the last injection day), the animals were sacrificed and the hearts were harvested and evaluated for necrosis using hematoxylin and eosin staining. In addition, Akt protein expression levels and BAX/BCL2 ratio were determined by western blotting. Our results showed that hesperidin decreased cardiac necrosis. In animals treated with hesperidin 100 mg/kg, Akt protein expression was increased, while the BAX/BCL2 ratio was significantly decreased. ECG changes were reversed in all groups 2 h following CO exposure, regardless of hesperidin administration. Overall, hesperidin decreased the deleterious cardiac effects of CO poisoning in rats.

Introduction
Carbon monoxide (CO) is a colorless and odorless toxic gas that competes with oxygen for binding sites of hemoglobin. It is considered the most common cause of poisoning-related mortality and morbidity worldwide (Goldstein 2008, Dindar Badem et al. 2019). CO is produced by incomplete combustion of fossil fuels and is found in motor vehicle exhaust emissions, poorly burning furnaces, charcoal burning and tobacco smoking (Satran et al. 2005, Mohamadpour et al. 2012, Eichhorn et al. 2018). Clinical manifestations of CO poisoning include injuries in the organs with great oxygen consumption, such as the brain and heart (Ghorbani et al. 2017, Tabrizian et al. 2017). Intoxication with CO causes myocardial infarction (MI), cardiomyopathy, tachycardia, dysrhythmia, hypotension, ischemia, and, in more severe cases, cardiac arrest (Goldstein 2008). Severity of cardiac poisoning is associated with the blood levels of carboxyhemoglobin (COHb) and duration of CO exposure (Kaya et al. 2016). Even a long time after CO poisoning, MI can occur, especially in patients with increased COHb concentrations (Kalay 2016).

Ischemia and myocardial injury are common in moderate to severe CO intoxication (Kaya et al. 2016). Data from human studies showed ischemic electrocardiogram (ECG) changes following CO poisoning, including T wave flattening or inversion, ST segment elevation and depression, QT prolongation, atrial fibrillation and intraventricular block (Weaver 2009). The most commonly reported ECG changes are T wave abnormality and S–T segment changes (elevation and depression) (Weaver 2009). Moreover, ventricular tachycardia and fibrillation were demonstrated following severe CO poisoning in rats (Weaver 2009).

Hesperidin belongs to a flavonoid subgroup of citrus flavonoids. It possesses a variety of anti-oxidant, anti-inflammatory, anti-aging, blood lipid and cholesterol lowering and...
anti-tumorigenesis properties (Parhiz et al. 2015, Pari et al. 2015, Bahramsooltani et al. 2019, Fouad et al. 2019). In addition, hesperidin has protective effects against cardiac ischemia/reperfusion (He et al. 2017). Furthermore, hesperidin has the ability to inhibit cardiac hypertrophy and fibrosis, oxidative stress and myocytes’ apoptosis induced by pressure overload, offering protection against cardiac dysfunction (Deng et al. 2013). Among various signaling proteins, protein kinase B (PKB, also known as Akt) appears to be a central player in metabolism regulation, cell survival, motility, and transcription as well as cell cycle (Fayard et al. 2005). Moreover, this protein is a key protein in pathways of myocardial salvation (Fayard et al. 2005).

In the current study, we evaluated the possible cardioprotective effects of hesperidin on ECG changes, necrosis, Akt protein expression levels and the apoptotic index BAX/BCL2 protein ratio, following acute CO poisoning in rats.

Materials and methods

Animals

Twenty male Wistar rats (200–250 g) were housed in the Animal Center of Zabol University of Medical Sciences, Zabol, Iran. They were kept under standard conditions (at 21–23 °C with 12 h/12 h light/dark cycles) and they had free access to food and water. The study was approved by the Animal Care Committee of Zabol University of Medical Sciences, Zabol, Iran (approval No. Zbmu.1.rec.1396.185).

Chemicals

Anti-Akt, anti-BAX, anti-BCL2, anti-β actin, and anti-rabbit secondary antibody were purchased from Cell Signaling, Beverly, MA. Hesperidin was purchased from Sigma-Aldrich (Berlin, Germany). Coomassie (Bradford, Renton, WA) protein assay kit was purchased from Thermo Scientific (Rockford, IL). Carbon monoxide capsule (99.999% purity) was obtained from Darman Gas (Tehran, Iran).

Experimental groups and study design

Animals were randomly divided into four groups (n = 5) as follows: normal saline-treated animals (control group), and three groups of rats treated with hesperidin (25, 50, and 100 mg/kg). Hesperidin doses were selected based on a pilot study and previously published reports (Maiti et al. 2009, Wasowski et al. 2012, Donato et al. 2014, Justin Thenmozhi et al. 2017, Qi et al. 2019). The animal model of CO-poisoning was applied as previously published (Hashemzai et al. 2016a, Hashemzai et al. 2016b, Tabrizian et al. 2017, Hashemzai et al. 2018, Tabrizian et al. 2018, 2019): all animals were anesthetized by intraperitoneal (ip) injections of ketamine (100 mg/kg) and xylazine (10 mg/kg) before exposure to CO and anesthesia was maintained during the experiments using the above-mentioned anesthetics at a dose which was half the initial one (Hashemzai et al. 2016a). Then, the animals were placed in an airtight Plexiglas container with entrance and exit taps. CO was delivered to the container to achieve a concentration of 3000 ppm required for intoxication. CO dose was selected based on our previous report (Shahsavand et al. 2012). Exposure lasted for 60 min. CO level in the exposure chamber was continuously monitored using a CO analyzer (TPI707 carbon monoxide analyzer, Korea). At the end of the 60-min CO exposure, animals were removed from the box and treated with intraperitoneal injections of normal saline (control group) or different doses (25, 50, and 100 mg/kg/day) of hesperidin, once a day, for 5 consecutive days according to our previous studies (Hashemzai et al. 2016a). On day 6, the animals were anesthetized using ketamine (100 mg/kg; ip) and xylazine (10 mg/kg; ip) and sacrificed. Following sacrifice, the hearts were immediately excised, fixed in formalin 10% for 24 h and stained with Hematoxylin and Eosin (H&E). For western blot analysis, the hearts were rinsed with saline solution and stored at −80 °C (Hashemzai et al. 2016a). About 200 mg from each heart was homogenized (at 1000 g for 10 min) in a buffer solution (50 mmol/L tris.HCl, pH 7.5, 150 mmol/L NaCl, and 1% SDS/protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)). The protein content of the supernatant was measured by the Bradford protein assay kit. Samples’ protein levels were identical. The samples were either used fresh, or stored at −70 °C.

To monitor the apoptotic pathway in heart tissue caused by CO-poisoning, as previously demonstrated (add refs here), Akt, BAX and BCL2 protein levels in heart samples were determined as follows: 10–20 μl of the tissue supernatant was loaded on SDS page wells and proteins were separated using gel electrophoresis. At the end of the electrophoresis procedure, proteins were transferred to polyvinylidene fluoride (PVDF) using a transfer buffer (25 mM glycine, 20% methanol, pH 8.0). The membrane was washed three times with washing buffer (5 min each time), in order to remove any unbound conjugate proteins. The membranes were subsequently treated with secondary antibody, washed thoroughly with Tris-buffered saline and Tween 20 (TBST) and visualized using 500–1000 μl enhanced chemiluminescence (Pierce, Rockford, IL). Finally, proteins levels were calculated using an image software (NIH Image J system, Bethesda, MD), following imaging in the dark and computer scanning (Tabrizian et al. 2017).

Carboxyhemoglobin level assessment

COHb levels were evaluated in order to confirm the animal model of CO-poisoning. For this purpose, immediately after CO exposure, blood samples were collected from the rats’ tails and blood levels of COHb were determined using a spectrophotometric method (Jenway 6305, Bibby Scientific Ltd., Staffordshire, UK) (Rodkey et al. 1979).

ECG and heart rate recording

Heart rate and ECG in lead I were recorded (Power Lab, AD Instrument, Bella vista, New South Wales, Australia) before and during CO exposure; also, similar measurements were done after removing the animals from the exposure chamber and exposing them to fresh air. The ECGs were analyzed for
ST segment changes (depression or elevation), T wave changes, AV block, pathologic Q waves and arrhythmia, including premature ventricular contractions (PVC), sick sinus syndrome (SSS), ventricular tachycardia, and fibrillation and atrial fibrillation (Mohamadpour et al. 2012, Tabrizian et al. 2019).

Statistical analysis
Data analysis was performed using SPSS version 11.5 (SPSS, Chicago, IL). Individual groups were assessed by Chi-square or Fisher’s exact test. Quantitative data were evaluated using One-way ANOVA followed by Tukey post hoc test. A p value < 0.05 was considered statistically significant.

Results

Carboxyhemoglobin levels following exposure to CO
Following exposure to CO for 60 min, blood levels of COHb reached toxic levels (73 ± 5 ppm, mean ± SD), confirming CO poisoning induction (Ghorbani et al. 2017).

Effects of hesperidin on ECG changes
At the beginning of CO poisoning, the animals’ heart rate increased up to 250–300 beats per minute (bpm) but after 30 min, their heart rate fell to < 200 bpm. CO poisoning caused arrhythmias including AV block, ST depression, PVT, and pathologic Q waves in all animals (Figure 1); however, 2 h after the discontinuation of CO exposure, all ECG parameters were restored to normal levels. ECG recordings showed that hesperidin did not decrease arrhythmias induced by CO poisoning.

Effects of hesperidin on histopathological findings
Histopathological findings in the heart samples were categorized into three grades. Grade 1 was defined as the presence of dispersed necrotic cells and/or lymphatic infiltration (Figure 2(A)); Grade 2 was defined as the presence of necrotic unifocal and/or bifocal areas (Figure 2(B)); and Grade 3 was defined as the presence of > 2 necrotic areas/sections (Figure 2(C) and Table 1).

H&E staining showed that while CO poisoning caused cardiomyocyte necrosis, hesperidin decreased necrosis foci. As shown in Table 1, in the control group, 4 out of 5 samples showed grade 3 necrosis. On the contrary, in the groups treated with hesperidin 25, 50, and 100 mg/kg, respectively 2, 1, and 0 out of 5 samples had grade 3 necrosis (Table 1). The reported reduction in necrotic foci seems to be dose dependent as the occurrence of grade 3 necrosis (i.e., samples that contained more than two necrotic foci) was reduced with increasing doses of hesperidin.

Effect of hesperidin on Akt protein level following CO exposure
Hesperidin 100 mg/kg significantly increased the expression of the anti-apoptotic protein Akt in comparison to the control group (p < 0.05). The lower doses of hesperidin (25 and 50 mg/kg) did not affect Akt protein expression, indicating that the effects of hesperidin on Akt expression are dose-dependent (Figure 3).

Effect of hesperidin on the BAX/BCL2 ratio following CO exposure
As shown in Figure 4, apoptosis (as depicted by the BAX/BCL2 ratio) was reduced by hesperidin in a dose-dependent manner compared to the control group. Administration of the high and the medium dose of hesperidin (100 and 50 mg/kg, respectively) resulted in a marked improvement, compared to the low dose of hesperidin (25 mg/kg).

Discussion
CO poisoning is rather common and damages human organs, especially the heart and brain which have greater demand for oxygen (Kaya et al. 2016, Ghorbani et al. 2017). Previously, we evaluated the potential therapeutic effects of different
chemicals and natural products against the toxic effects of CO in rats’ heart and brain (Tabrizian et al. 2017, Hashemzaei et al. 2018, Tabrizian et al. 2018, Bagheri et al. 2019). Hesperidin (5, 7, 3-trihydroxy-4'-methoxyflavanone 7-rhamnoglucoside) belongs to a class of flavonoids called flavanones and is found mainly in citrus fruits; this compound exerts various pharmacological/biological activities (Roohbakhsh et al. 2014, Iranshahi et al. 2015, Roohbakhsh et al. 2015) and is known for its protective effects on cardiomyocytes following ischemia/reperfusion and acute MI (Garg et al. 2018, Tong et al. 2018).

In the present study, hesperidin reduced cellular infiltration and necrosis in rat cardiomyocytes. Histopathological evaluation of CO-poisoned rat hearts showed that in the control group, 4 cases (out of 5) presented grade-3 injury, while in animals treated with the high dose of hesperidin (100 mg/kg), no case of grade-3 injury (i.e. 0 out of 5) was observed (Table 1). Moreover, the current study showed that cardiomyocyte apoptosis, as depicted by the apoptotic index BAX/BCL2 protein ratio, was significantly decreased in hesperidin-treated groups, compared to the control group. Moreover, this effect seemed to be dose-dependent as the BAX/BCL2 ratio gradually decreased with increasing doses of hesperidin.
with hesperidin 100 mg/kg having the most marked results (Figure 4).

Akt protein is a pro-survival protein and its increase in cardiomyocytes reflects improvement of cell survival. The present study showed that high doses of hesperidin increased Akt protein expression in rats’ myocardial tissue. It is known that activation of the PI3/kinase pathway enhances cell survival in cardiomyocytes (Li et al. 2016). Following activation of PI3/Akt, expression of the pro-apoptotic proteins, including BAX and caspase-3, decreases along with the decrement of the number of apoptotic cardiomyocytes (Chen et al. 2016). Members of the BCL2 family are major regulators of the mitochondrial pathway of apoptosis (Dejean et al. 2006, Machado et al. 2018), which can be classified into two functionally distinct groups, the anti-apoptotic and the pro-apoptotic components (Martinou and Youle 2011, Machado et al. 2018). BCL2, an anti-apoptotic protein, preserves mitochondrial integrity and protects against cell death (Hockenbery et al. 1993, Machado et al. 2018). Conversely, BAX, a pro-apoptotic protein which is expressed abundantly and selectively during apoptosis, promotes cell death (Machado et al. 2018). According to our results, hesperidin 25 and 50 mg/kg favorably modified the BAX/BCL ratio, while had no significant effect on Akt expression.

CO poisoning is thought to cause cardiomyocyte apoptosis via two major mechanisms: (1) binding to cytochrome C oxidase in the electron chain, resulting in asphyxiation at the cellular level and (2) inducing the degranulation of neutrophils and thus increasing the circulatory levels of myeloperoxidase and reactive oxygen species. These cause oxidative stress, which negatively affects cellular components and organelles (Rezaee et al. 2014, Garg et al. 2018). These effects lead to activation of the pro-apoptotic BAX and BAD proteins, as well as inhibition of the anti-apoptotic BCL2 protein (Hashemzaei et al. 2016a, 2016b, Ghorbani et al. 2017, Garg et al. 2018).

There is currently evidence that short-term administration of different doses (100 and 200 mg/kg) of hesperidin could decrease the BAX/BCL2 ratio and apoptosis, following ischemia/reperfusion (Agrawal et al. 2014, Kapoor and Kakkar 2014, Varshney et al. 2015, Li et al. 2016). Our study confirmed that hesperidin decreases the apoptotic index BCL2/BAX ratio in CO-poisoned rat cardiomyocytes.

Hesperidin decreases myocardial injury following ischemia/reperfusion and improves blood flow in ischemic myocardial regions (Gandini et al. 2001, Rong et al. 2013, Agrawal et al. 2014). Agrawal et al. reported that pretreatment with hesperidin in an animal model of cardiac ischemia/reperfusion, significantly decreased oxidative stress burden (as reflected by decreased levels of thiobarbituric acid reactive substance), restored lactate dehydrogenase activity towards normal levels and exerted anti-apoptotic effects (by down-regulation of BAX and up-regulation of BCL2) (Agrawal et al. 2014). It is possible that in the current study, the favorable modulatory effects of hesperidin on BAX/BCL2 ratio and myocardial necrosis, occurred partly through oxidative stress amelioration.

In conclusion, the current study showed that hesperidin reduces cardiomyocyte necrosis and decreases the apoptotic index BAX/BCL2 ratio in CO-exposed animals. Furthermore, high doses of hesperidin increased myocardial Akt protein levels, compared to the control group. Animal ECG parameters and arrhythmia occurrence were not modified by hesperidin. However, all ECG changes in CO-exposed rats were reversed 2 h post-exposure.

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Disclosure statement

Authors declare that they have no conflict of interest.

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