EXPERIMENTAL STUDY

The protective effect of betanin and copper on heart and lung in end-organ ischemia reperfusion injury

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ABSTRACT

OBJECTIVES: Betanin and copper sulphate have been previously indicated as beneficial agents for ischemia/reperfusion (I/R) as antioxidant compounds in various models. We investigated whether betanin and copper have any protective effects on the heart and lung against I/R injury in rats.

METHODS: Spraque-Dawley rats were assigned in groups: Sham (laparotomy only), control (I/R only), betanin treatment (100 mg/kg of betanin administered intraperitoneally (i.p.) 60 minutes before I/R) and copper sulfate treatment group (0.1 mg/kg/day copper sulfate i.p. for 7 days before I/R). Ischemia was induced by clamping the aorta between the left renal artery and aortic bifurcation for 45 minutes. After 48-hour reperfusion, the rats were sacrificed and heart/lung tissues were harvested. Malondialdehyde (MDA), myeloperoxidase (MPO), interleukin 6 (IL-6) levels were determined. Apoptosis was determined via TUNEL assay.

RESULTS: MDA, MPO, IL-6 levels and apoptotic cells were significantly increased in the I/R group. In both treatment groups, MDA and MPO levels were decreased. IL-6 was significantly decreased in response to betanin administration in the heart, but not in the lung; copper had no effect in either area. The numbers of apoptotic cells were significantly decreased in both treatment groups.

CONCLUSION: Betanin and copper may have protective effects on I/R injury in the heart and lung in rats (Fig. 6, Ref. 39).

KEY WORDS: ischemia reperfusion injury, betanin, copper, antiinflammation, antioxidant, rat.

Introduction

Abdominal aortic surgery could result in mortality and morbidity by causing ischemia reperfusion (I/R) injury in remote organs such as heart and lung after the aortic clamp removal (1).

I/R injury causes remote organ damage which consequently leads to a release of both, reactive oxygen species (ROS) and molecules related to inflammation from ischemic tissues (2, 3).

It has been reported that as a result of I/R damage, ROS are released from neutrophils and macrophages. These free radicals then may lead to lipid peroxidation of cellular membranes, increased excitatory amino acids and destruction in nucleic acids and enzymes (4, 5). Increase in both inflammation-associated molecules and ROS-caused apoptosis (6, 7). Apoptosis is one of the main consequences of I/R injury (8). Therefore, targeting apoptosis by lowering the amount of ROS and inflammation are one of the attractive therapeutic approaches to prevent heart and lung failure (7, 9).

In recent years, studies with betalains have attracted attention. Red beet root (Beta vulgaris L.) is a good source of red pigments, called betalains (10). Betanin (betanidin 5-O-β-D-glucoside, red) makes up more than 75 % of the total betalain component (11). The betanin molecule includes phenolic and cyclic amine groups which are good electron donors, suggesting that these pigments have high antioxidant properties against I/R injury (12). Furthermore, they can reduce oxidative stress, inflammation and reduce associated disorders such as atherosclerosis, hypertension and cancer in addition to I/R injury (13–15). In addition to betanin, a low-dose copper sulfate administration has been proposed to have potential anti-inflammatory, anti-proliferative and antioxidant properties (16, 17). Therefore, this compound may also present with some cardioprotective qualities (18).

Malondialdehyde (MDA) is one of the end products of lipid peroxidation and has been widely used as an oxidative stress...
marker (19). Myeloperoxidase (MPO) is released from neutrophils and monocytes to react with $\text{H}_2\text{O}_2$ in the injured tissue, which displays powerful pro-oxidative and pro-inflammatory actions (20). Interleukin 6 (IL-6) is one of the leading inflammatory mediators that is released quickly in response to acute I/R (21).

The protective properties of betanin and copper sulphate on I/R injury on the heart and lungs have been poorly investigated up to now. In this study, we investigated whether betanin and copper sulphate had any anti-inflammatory and anti-apoptotic effects on the heart and lung in I/R-induced injury.

Materials and methods

Animals and study groups

The study was approved by the Institutional Ethics Board with a decision number 2016-107. Twenty-eight healthy adult male Spraque-Dawley rats (Rattus norvegicus) weighing 250–350 g were used for the study. All rats were kept on an ad libitum rat feed and water under optimal conditions in accordance with the protocol for ethical care of laboratory animals.

Rats were divided into 4 groups. Sham group (Group 1; n = 7) underwent only laparotomy without aortic clamping. The control group (Group 2; n = 7) underwent only aortic clamping procedure after laparotomy. In the betanin group (Group 3; n = 7), a dosage of 100 mg/kg of betanin (Tokyo chemical industry CO., Ltd., Japan; cat# B0397) was administered intraperitoneally (i.p.) 60 minutes before laparotomy and aortic cross clamping. Copper sulfate group (Group 4, n = 7) received copper (II) sulfate (Sigma, cat# C2284) i.p. at a dose of 0.1 mg/kg/day for 7 days before laparotomy and aortic cross clamping.

Operative procedure and technique

Rats were anesthetized using 50 mg/kg ketamine HCl (Ketalar vial 50 mg/ml, Pfizer, NY, USA) and 10 mg/kg xylazine hydrochloride (Rompun 2 %, Bayer, Turkey), i.p. Anesthesia was maintained with intermittent doses of ketamine. Heparin was administered i.p. at a dose of 400 IU / kg to all rats before the procedure. Standard midline laparotomy was performed after the surgical field had been cleared. Then the abdominal aorta was exposed via transperitoneal approach.

Ischemia was induced in groups 2, 3 and 4 by clamping the aorta for 45 minutes with mini aneurysm clips, just distal to the left renal artery, and proximal to the aortic bifurcation. Body temperatures of the animals were maintained constant by using a heating lamp. At the end of the surgical procedure, the abdominal wall was sutured with 5/0 polypropylene suture in all rats. After 48 hours, all animals were anesthetized with 100 mg/kg phenobarbital and sacrificed. No mortality or morbidity was seen during the experiment.

Biochemical analyses

Tissue preparation

Heart and lung tissues were harvested, samples were allocated to be washed with cold PBS and kept at −80 ºC for enzyme-linked immunosorbet assay (ELISA) and also fixed with 10% formalin, following paraffin mounting for the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay.

A 10-mg mass of heart or lung tissue was homogenized with a mechanical homogenizer in PBS buffer including protease inhibitors. Following centrifugation at 1000 rpm to remove tissue debris, total protein concentrations of the samples were determined using the Bradford assay.

Determination of MDA concentration

Tissue MDA levels were determined by a previously established protocol (22). Briefly, MDA reacts with thiobarbituric acid (TBA) at low pH to produce a pinkish-red colored chromogen. The concentration of MDA was determined spectrophotometrically using a standard curve at 532 nm. The results are given as μg/g. For the standard, tetramethoxypropane (Sigma T9889) was prepared and used (22).

Determination of MPO activity in the heart and lung

MPO activity can be measured in tissues by spectrophotometric assays using hydrogen peroxide and o-diamisidine dihydrochloride as substrates as previously described (23). Optical density was adjusted to kinetic mode and measured at 460 nm for 8 minutes.

Determination of IL 6 concentration in the heart and lung

The concentration of IL 6 was determined according to the manufacturer’s recommendations (Affymetrix eBioscience, Cat# BMS625, CA, USA). Briefly, each sample and standards were added into wells in duplicates. Biotin conjugate was then added and incubated at room temperature for 2 hours. After washing the wells six times, streptavidin-HRP was included and incubated for 1 hour. After the washing, tetramethyl benzidine (TMB) substrate solution was added and the concentration of IL 6 for each sample was measured at 620 nm. The concentration of IL 6 was expressed as pg/mg in total protein.

TUNEL assay

Fixed heart and lung tissues in paraffin blocks were sectioned at a thickness of 10 μm. TUNEL assay was performed using an in situ apoptosis detection kit according to the manufacturer’s recommendation (Abcam, Cat# ab206386, MA, USA). In brief, diaminobenzidine (DAB) reacts with the HRP-labeled sample to generate brown color at the site of DNA fragments. Methyl green was used as a counterstain. DNase I was applied to generate a positive control. The sections were permeabilized with proteinase K for 20 minutes and endogenous peroxidase activity was suppressed with 3 % H$_2$O$_2$ for 5 minutes. The images of stained sections were obtained using BestScope (BLM-280, Beijing, China) microscope camera at 400 magnifications for further analyses.

Cells were counted at ×100 magnification in ten separate arbitrary fields in each tissue section. Cells having brown-stained nuclei were considered apoptotic. The percentage of the TUNEL-positive cells was expressed as the apoptotic index, as previously reported (24).

Statistical analyses

MDA, MPO and IL 6 levels for biochemical analyses and TUNEL assay for the analysis of apoptosis were performed for
all groups. Statistical analyses were performed using SPSS software (SPSS Inc., USA). Appropriate data were presented as mean ± standard deviation. One-way ANOVA and post hoc for multiple comparison tests were used to compare the groups. The criterion for statistical significance was set at * p < 0.05, ** p < 0.01, or *** p < 0.001.

Results

The effect of betanin and copper on MDA, MPO and IL-6 in the I/R injured heart and lung

When compared to sham group, I/R injury was successfully established in the control group, which was indicated by signifi-

Fig. 1. MDA levels (μg/g) in the heart and lung. Heart and lung tissues from each group were collected and homogenized. The concentration of MDA was determined spectrophotometrically in the heart (A), and lung (B). Comparison was made with one-way ANOVA and post hoc. Data represent means ± S.D. * p < 0.05, ** p < 0.01 and *** p < 0.001.

Fig. 2. Relative MPO activity in the heart and lung. Heart and lung tissues from each group were collected and homogenized. The activity of MPO was determined in the heart (A), and lung (B). Comparison was made with one-way ANOVA and post hoc. Data represent means ± SD. * p < 0.05, ** p < 0.01 and *** p < 0.001.

Fig. 3. IL-6 levels (pg/mg) in the heart and lung. Heart and lung tissues from each group were collected and homogenized. The concentration of IL-6 was determined spectrophotometrically in the heart (A), and lung (B). Comparison was made with one-way ANOVA and post hoc. Data represent means ± S.D. * p < 0.05, ** p < 0.01 and *** p < 0.001.
cant increases in MDA, MPO and IL-6 levels, both in the heart and lung (Figs 1–3).

Both betanin and copper treatment significantly reduced MDA levels in the heart relative to the control (p < 0.001 for both) (Fig. 1). When compared to copper, betanin had a significantly higher reducing effect on MDA in the heart (p < 0.05) (Fig. 1A).

As for MDA levels in the lung, we found a similar trend as seen in the heart, in which both betanin and copper significantly reduced MDA levels relative to Group 2 (control; p < 0.01 and p < 0.05, respectively). When compared to copper, betanin had a more reducing effect in the lung, but it was not significant (Fig. 1B).

Both betanin and copper treatments had a similar reducing effect on MPO levels relative to the control in the heart (p < 0.001 for both) (Fig. 2A). Likewise, in the lung, both these agents reduced MPO levels (p < 0.05 and p < 0.01, respectively) (Fig. 2B).

Betanin lowered IL-6 levels in the heart (p < 0.05) (Fig. 3A), but the decrease in IL-6 in the lung was not significant (Fig. 3B). Copper, on the other hand, did not significantly change IL-6 levels either in the heart or in the lung (Fig. 3A and B).

**Discussion**

As a result of the I/R injury following cardiovascular system surgeries, the heart and lung functions can be depressed. Cellular pathophysiologic mechanisms underlying myocardial and lung I/R injuries are diverse, including ion accumulation, dissipation...
of mitochondrial membrane potential, increased ROS formation, apoptosis and/or autophagy, immune activation and neutrophil infiltration, and endothelial dysfunction (25). Since the reason behind the I/R injury in the heart and lung is complex, interventions require a spectrum of targets, mainly in the antioxidant and anti-inflammatory pathways (26–29). The generation of free radicals, especially in the first few minutes of myocardial and lung reperfusion can harm cellular lipids, proteins, and nucleic acids and ultimately lead to cell death.

The present study shows that betanin and copper may have some antioxidant, anti-inflammatory and anti-apoptotic qualities. To test whether betanin or copper treatment had any oxidative stress decreasing effects in the I/R injury, we first determined the heart and lung MDA levels. We observed that both betanin and copper significantly prevented oxidative stress and lipid peroxidation. The effects of these agents were more noticeable in the heart than in the lung. Antioxidative and anti-inflammatory properties of betanin and copper were further evaluated by determining the activity of MPO enzyme. It has been suggested that targeting MPO and its downstream inflammatory pathways represent an attractive therapeutic intervention for the I/R injury. Our results showed that either a single dose of 100 mg/kg betanin or that of 0.1 mg/kg/day copper for 7 days significantly reduced the activity of MPO in our I/R model in the heart and lung in rats. Betanin was also reported to have an inhibitory action on lipid hydroperoxide production in human LDL caused by MPO/nitrite-induced oxidation (30). Moreover, a recent study has reported that betanin reduces oxidative damage and decreases MPO activity in isoproterenol-induced acute myocardial infarction (31).

Whether copper is having any protective effect on I/R injury in the heart and lung has been very poorly studied. Copper has been reported to be directly involved in the inhibition of MPO activity in vitro (32). The loss of copper after myocardial ischemia has been demonstrated. In this same study, the authors have proposed that copper supplementation protects the heart from the ischemic toxicity (33). Copper might have comparable roles in the lung as well as in the heart. Copper levels are observed to decrease in damaged lung tissue (34). Associated with this, in another study, due to copper deficiency, the level of MPO was significantly increased in the lungs of the rats (35).

The beneficial effects of red beet extract treatment by reducing pro-inflammatory factors, including IL-6 in human with osteoarthritis and gentamicin-induced nephrotoxicity have been reported; however, the question as to which specific compound in that extract is having these effects remains unanswered (36, 37). The effects of betanin on heart and lung I/R has been very poorly studied so far. Therefore, this study provides some novel findings specifically on betanin in the I/R injury of the heart and lung. We checked whether betanin and copper administration influenced IL-6-mediated inflammation following the I/R injury. According to our results, betanin, but not copper had significant anti-inflammatory roles by reducing IL-6 for the heart, suggesting that the different pathways that these agents act play a role in I/R injury. Additionally, neither betanin nor copper displayed this quality for the lung, indicating organ-specific actions of the agents. It is possible that betanin had an inhibitory function on neutrophil infiltration and/or function (38) both in the heart and lung, and it has anti-inflammatory roles in the heart by reducing the levels of IL-6, but that was not effective in the lung. The reason why betanin was ineffective for the lung may be due to the fact that it had no influence on macrophage action in the lung.

Apoptosis is the major consequence of myocardial and lung I/R injury. We tested whether betanin and copper had any anti-apoptotic activities in the heart and lung using the TUNEL assay. We found that these agents significantly reduced the number of apoptotic cells caused by I/R process both in the heart and lung. It has been reported that betanin prevents apoptosis by inhibiting CYP 3A2 expression and prevented mitochondrial damage (39). These anti-apoptotic actions of these agents on the heart and lung might stem from their antioxidant properties.
Conclusion

Interactions of ROS and antioxidant agents play an important role in a variety of pathologic processes and are thus investigated on different models. Most of those agents have low toxicity and high therapeutic index and potential for clinical application. In our study we found that betanin and copper may have valuable cytoprotective roles in the heart and lung in the I/R injury. We suggest that the protective actions of betanin and copper are associated with antioxidant, anti-inflammatory and anti-apoptotic properties. Nevertheless, further studies should be performed for their mechanisms of action.

References


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