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Research Article

The protective effect of betanin and copper on spinal cord ischemia–reperfusion injury

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Context: Both copper and betanin have been implicated as having significant bioactivity against ischemic damage in a variety of experimental and clinical settings. The aim of this study is to investigate whether betanin and copper have any protective effect on spinal cord in an ischemia–reperfusion (I/R) model in rats.
Design: Spraque-Dawley rats were used in four groups: Sham group ($n = 7$), control group (laparotomy and cross-clamping of aorta, $n = 7$), betanin treatment group (dosage of 100 mg/kg of betanin administered intraperitoneally (i.p.) 60 min before laparotomy, $n = 7$), copper sulfate treatment group (administered copper sulfate i.p. at a dose of 0.1 mg/kg/day for 7 days before laparotomy, $n = 7$). Malondialdehyde (MDA), glutathione (GSH) levels, myeloperoxidase (MPO) and superoxide dismutase (SOD) activity were measured. Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay was also performed to evaluate apoptosis.
Setting: Kafkas University, Faculty of Medicine, Kars, Turkey.
Results: I/R injury was successfully demonstrated with the surgical model. Betanin and copper treatment significantly decreased MDA levels, MPO activity and the number of apoptotic cells in the spinal cord. Betanin and copper treatment significantly increased GSH levels. Copper treatment significantly increased SOD activity, whereas betanin was not as effective. Apoptotic cells were significantly decreased in both treatment groups.
Conclusion: I/R injury of the spinal cord can be successfully demonstrated by aortic clamping in this surgical model. Betanin/Copper sulphate has ameliorative effects against operative I/R injury. Low toxicity of those agents makes them ideal targets for clinical research for this purpose.

Keywords: Spinal cord, Ischemia–reperfusion injury, Betanin/Copper, Antioxidant, Anti-inflammatory

Introduction

Spinal cord ischemia after aortic surgery is a rare but devastating complication. Its incidence was reported in an early landmark study as 0.25% in over 3000 abdominal aortic operations.[1] Generally, the clinical picture of this complication is concurrent with anterior horn damage, including symptoms of motor and sensory deficits in lower extremities and bladder-bowel dysfunction. In a case series of 18 patients with spinal cord damage after abdominal aortic surgery, the prognosis was reported to be poor with eight patients not surviving 30 days, five patients undergoing urgent lumbar drainage with no clinical effect.[2]

I/R model in rat via laparotomy and abdominal aorta cross-clamping, often just distal to the left renal artery, proximal to the aortic bifurcation is an established model to study end-organ (lung, heart and kidney) and spinal cord damage.[3–5]

Betanin and copper sulphate has been studied as ameliorating agents against radical oxygen species (ROS) damage in various settings. Betanin is the main

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component of red beetroot pigment group, which is collectively called betalains. Betanin molecule has cyclic and phenolic amine groups which make it a very good electron donor and ROS scavenger in cases of oxidative stress. Copper on the other hand mainly exerts its effects via NO system, vasodilation and it is a coenzyme for SOD. Also both betanin and copper have been shown to have anti-neoplastic effects.[6–10] Their neuroprotective effects in an I/R setting is poorly understood, but both agents are promising in terms of clinical use with a good safety profile.

Malondialdehyde (MDA) is a reliable marker to determine oxidative stress in clinical situations being a product of enzymatic degradation and lipid peroxidation of polyunsaturated fatty acids.[11] Glutathione (GSH) levels,[12,13] myeloperoxidase (MPO)[13] and superoxide dismutase (SOD) activity[13] were measured as oxidative stress and inflammation markers in spinal cord tissue as well. Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay was also performed to evaluate apoptosis.[14]

In this study, our objective is to investigate effects of betanin and copper sulphate on spinal cord tissue in an I/R model in rats with primary outcomes of MDA and GSH concentration and MPO and SOD activity, apoptosis via TUNEL assay.

Materials and methods

Animals and study groups

The study was approved by the Institutional Ethics Board with a decision number 2016-141. Twenty-eight adult male healthy Spraque-Dawley (*Rattus Norvegicus*) rats weighing 250 to 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 lab animals.

Rats were assigned in four groups: Sham group (n = 7, laparotomy only), Control group (n = 7, I/R only), Betanin group (n = 7, 100 mg/kg of betanin (Tokyo chemical industry CO., Ltd., Japan; cat# B0397) intraperitoneally (i.p.) 60 min before I/R), Copper sulfate group (n = 7 copper(II) sulfate (Sigma, cat# C2284) solution given i.p. at a dose of 0.1 mg/kg/day for 7 days before I/R).

Operative procedure and technique

Rats were anesthetized using 50 mg/kg ketamine HCI (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. Standard midline laparotomy was performed after the surgical field was cleared, and the abdominal aorta was explored with transperitoneal approach.

Heparin was administered i.p. at a dose of 400 IU/kg to all rats before the procedure. Ischemia was induced by cross-clamping the aorta with mini aneurysm clamps, just distal to the left renal artery and proximal to the aortic bifurcation for 45 min. Body temperatures of the animals were maintained constant by a heating lamb. All the cross-clamp groups were let to reperfuse for 48 h after the 45-minute ischemia. After 48 h, all animals were anesthetized by 100 mg/kg phenobarbital and sacrificed.[15] Lumbar spinal cord was harvested between lower border of L2 and upper border of L5 and allocated for homogenization (bottom half) and formaldehyde fixation (upper half). No mortality and unexpected morbidity were seen during the experiment.

Neurologic assessment

Tarlov score was recorded for each subject at post-operative 24th and 48th hours. Scoring is as follows: 0 = no voluntary hind-limb movement, 1 = movement of joints perceptible, 2 = active movement but not able to sit without help, 3 = able to sit but not able to hop, 4 = weak hop, 5 = complete recovery of hind-limb function. A medical doctor blinded to the experimental groups performed the neurologic assessments.

Biochemical analyses

Tissue preparation

Spinal cord tissues were harvested, samples were allocated to be washed with cold phosphate-buffered saline (PBS) and kept at −80°C for enzyme-linked immunosorbent assay and also fixed with 10% formalin, following paraffin mounting for the TUNEL assay.

Ten milligrams of spinal cord tissue was homogenized by a mechanical homogenizer in PBS buffer including protease inhibitors. Samples were centrifugated at 1000 rpm to remove tissue debris, total protein concentrations of the samples were determined using the Bradford assay.

Determination of MDA concentration in the spinal cord

Tissue MDA levels were determined by a previously established protocol.[16] Briefly, MDA reacts with tiobarbituric acid at low pH to produce a chromogen. The concentration of MDA was determined spectrophotometrically using a standard curve at 532 nm. For the standard, tetramethoxypropane (Sigma T9889) was prepared and used.

Determination of MPO activity in the spinal cord

MPO activity can be measured in tissues by spectrophotometric assays using hydrogen peroxide and

o-dianisidine dihydrochloride as substrates as previously described in.[17] Optical density was adjusted to kinetic mode and measured at 460 nm for 8 min.

Determination of SOD activity in the spinal cord

Tissue homogenization and the activity of SOD were determined according to the manufacturer's recommendations (706002; Cayman Chemicals, MI, USA). The kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The absorbance of the standards and the samples were measured at 450 nm. The final volume of each reaction was 230 ml. The activity of SOD was expressed as units/ml. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The samples and the standards were assayed in triplicate.

Determination of GSH concentration in the spinal cord

The concentration of GSH was determined according to the manufacturer's recommendations (SunRed, 201-11-5134, China). Samples standards GSH-antibody, Streptavidin-HRP were added into each well and incubated for 1 h at 37°C. After washing each well six times, chromogen solution was added and incubated for 10 min at 37°C. Reactions were stopped by adding stop solution and measured at 450 nm via a plate reader.

TUNEL assay

Fixed spinal cord 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 (Cat# ab206386, Abcam, MA, USA). In brief, diaminobenzidine reacts with the HRP-labeled sample to generate a brown color at the site of DNA fragments. Methyl green was used as counterstain. DNase I was applied to generate a positive control. Sections were permeabilized with proteinase K for 20 min and endogenous peroxidase activity was suppressed with 3% H_2O_2 for 5 min. The images of the stained sections were obtained using BestScope (BLM-280, Beijing, China) microscope camera at ×400 magnification for further analyses.

Cells were counted at ×100 magnification in 10 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.[18]

Statistical analysis

Statistical analysis was performed using SPSS 24.0 software (SPSS Inc., USA). Kolmogorov–Smirnov test was performed to assess the distribution of continuous variables. Non-parametric testing was done with Mann–Whitney *U* or Kruskal–Wallis test as appropriate. Repeated measures were taken for intergroup analysis.

Results

The effect of betanin and coper on MDA, MPO, SOD and GSH in the I/R injured spinal cord

Spinal cord I/R injury was successfully demonstrated after application of the surgical model ($n = 7$ for all groups), evidenced by increased MDA and MPO activity levels ($P = 0.001$ for both), decreased SOD activity and GSH ($P = 0.002$ and $P = 0.001$, respectively) when we compared the control group against sham surgery group (Figs 1–4).

MDA levels and MPO activity were found to be decreased in both betanin and copper groups when compared to the control group ($P = 0.001$ and $P = 0.007$ for betanin group, and $P = 0.001$ and $P = 0.002$ for copper group, respectively, Figs 1 and 2). SOD activity was increased in copper group ($P = 0.001$) but not in Betanin group when compared to controls ($P = 0.128$) (Fig. 3). Betanin and copper groups both had higher GSH levels when compared to the control group ($P = 0.001$, Fig. 4).

When we compared copper and betanin groups, MDA levels and MPO activity were found to be comparable ($P = 0.805$ and $P = 0.456$, respectively). SOD activity and GSH levels were found to be significantly increased

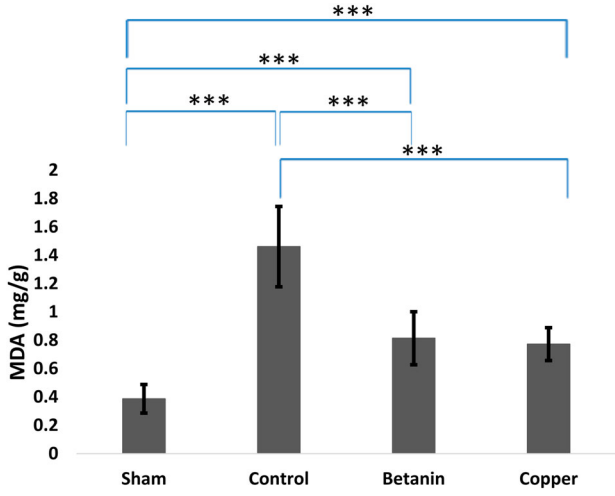

Figure 1 MDA levels (µg/g) in the spinal cord. Spinal cord tissues from each group were collected and homogenized. The concentration of MDA was determined spectrophotometrically in the spinal cord. Data represent means ± SD. Mann–Whitney *U* test. Control/betanin/copper groups versus sham P = 0.001 for all; betanin or copper groups versus control P = 0.001, respectively. *n* = 7 for all groups. *P < 0.05, **P < 0.01 and ***P = 0.001.

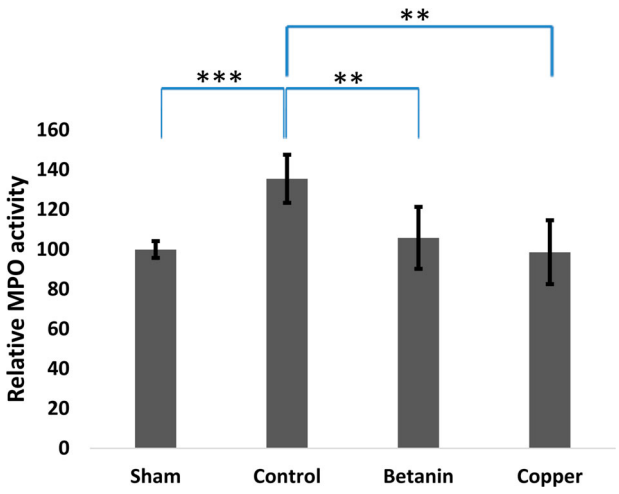

Figure 2 Relative MPO activity in the spinal cord. Spinal cord tissues from each group were collected and homogenized. The activity of MPO was determined in the spinal cord. Data represent means ± SDs. Mann–Whitney *U* test. Control versus sham P = 0.001; betanin and copper groups versus control group P = 0.007 and P = 0.002, respectively. *n* = 7 for all groups. *P < 0.05, **P < 0.01 and ***P = 0.001.

in copper group when compared to betanin group (P = 0.002 and P = 0.007, respectively, Figs. 1–4).

Betanin group was significantly improved regarding increased GSH and decreased MPO when compared to sham group (P = 0.620 and P = 0.209, respectively). Copper group was significantly improved in terms of increased SOD activity and decreased MPO when compared to sham group (P = 0.128 and P = 0.535, respectively, Figs 2–4).

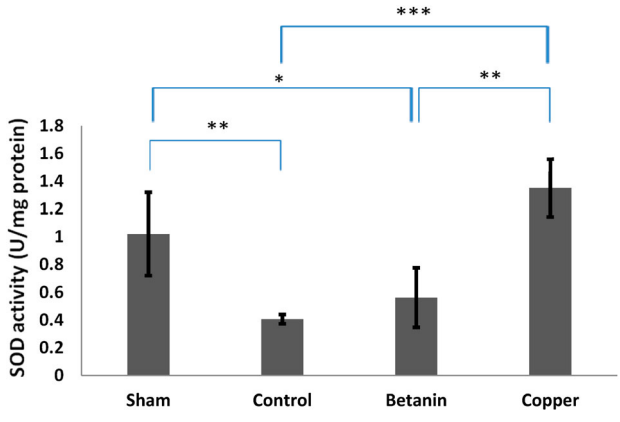

Figure 3 SOD activity (pg/mg) in the spinal cord. Spinal cord tissues from each group were collected and homogenized. The activity of SOD was determined spectrophotometrically in the spinal cord. Data represent means ± SD. Mann–Whitney *U* test. Control versus sham P = 0.002; betanin and copper groups versus control group P = 0.128 and P = 0.001 for both; copper versus betanin P = 0.002. *n* = 7 for all groups. *P < 0.05, **P < 0.01 and ***P = 0.001.

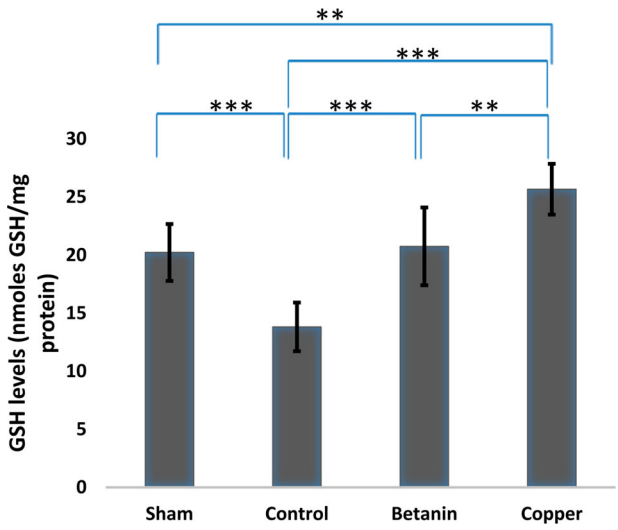

Figure 4 GSH levels (µg/g) in the spinal cord. Spinal cord tissues from each group were collected and homogenized. The concentration of GSH was determined spectrophotometrically in the spinal cord. Data represent means ± SD. Mann–Whitney *U* test. Control versus sham P = 0.001; betanin and copper groups versus control group P = 0.001 for both; copper versus betanin P = 0.007. *n* = 7 for all groups. *P < 0.05, **P < 0.01 and ***P = 0.001.

The effects of betanin and copper on apoptosis in the I/R injured spinal cord

Apoptotic index was found to be lower in the intervention groups, when compared to spinal I/R group (Figs 5 and 6). In the I/R group, the number of apoptotic cells significantly increased (P = 0.001) in the spinal cord tissues relative to the sham groups. Betanin and copper treatment decreased the number of apoptotic cells in the spinal cord relative to the control group (P = 0.026 and P = 0.008, respectively). The number of apoptotic cells was comparable between betanin and copper treatment groups. The number of apoptotic cells in betanin and copper groups versus sham group was found significantly lower (P = 0.001 for both). The quantification of apoptotic cells were presented in the spinal cord in Fig. 6.

The effects of betanin and copper on Tarlov scores in the I/R injured spinal cord

Tarlov scores were decreased in control group versus sham surgery in both 24th and 48th hours (1.14 ± 1.87 and 1.43 ± 1.81 vs 5.0 ± 0.0, P = 0.003). Betanin did not result in improvement when compared against control group but copper sulphate had significant improvement in both time points (2.71 ± 1.89 vs 1.14 ± 1.87 and 2.71 ± 1.89 vs 1.43 ± 1.81 P = 0.076 and P = 0.190 for betanin in both 24th and 48th hours, 3.86 ± 1.95 vs 1.14 ± 1.87 and 4.0 ± 1.73 vs

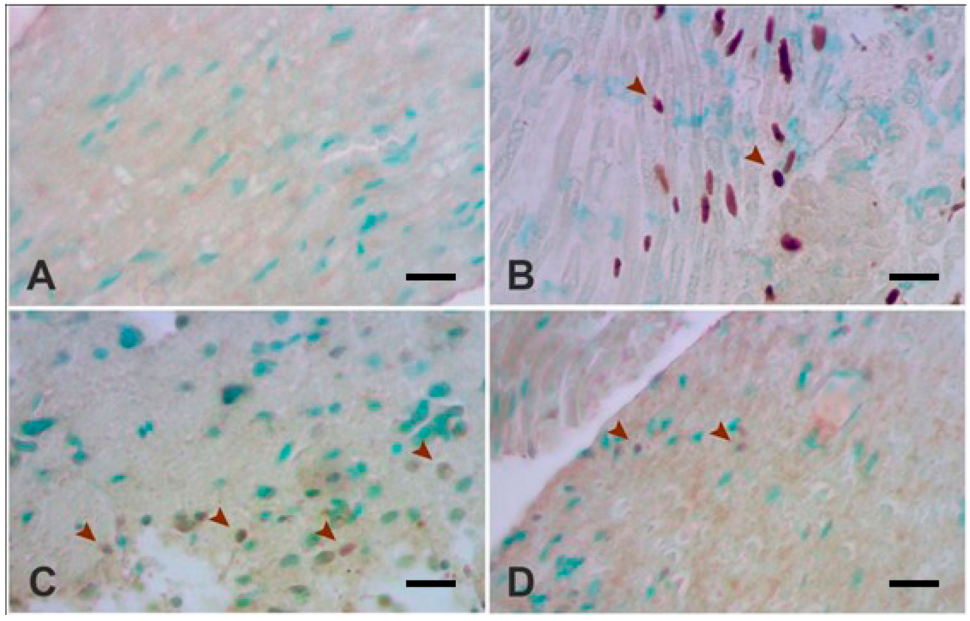

Figure 5 Detection of apoptosis in the spinal cord. Detection of apoptotic cells using TUNEL staining (A–D). TUNEL-positive cells were counter stained with methyl green. (A) Sham, (B) Control, (C) Betanin treatment, (D) Copper sulphate treatment. TUNEL-positive cells were counter stained with methyl green. Bar: 50 µm. *n* = 7 for all groups. *P < 0.05, **P < 0.01 and ***P = 0.001.

1.43 ± 1.81, P = 0.026 and P = 0.038 for copper in both 24th and 48th hours). Copper group was comparable in Tarlov scores when compared to sham group (P = 0.383 for both 24th and 48th hours). Tarlov scores did not differ significantly between betanin and copper treatment groups (P = 0.234 and P = 0.146, for 24th and 48th hours) (Figs 7–8).

Discussion

Spinal cord I/R injury after abdominal aortic surgery is a serious complication with a poorly understood mechanism and we lack evidence-based guidelines addressing this issue specifically. In this study, we were able to demonstrate spinal ischemia–reperfusion successfully using the established model, which closely mimics the

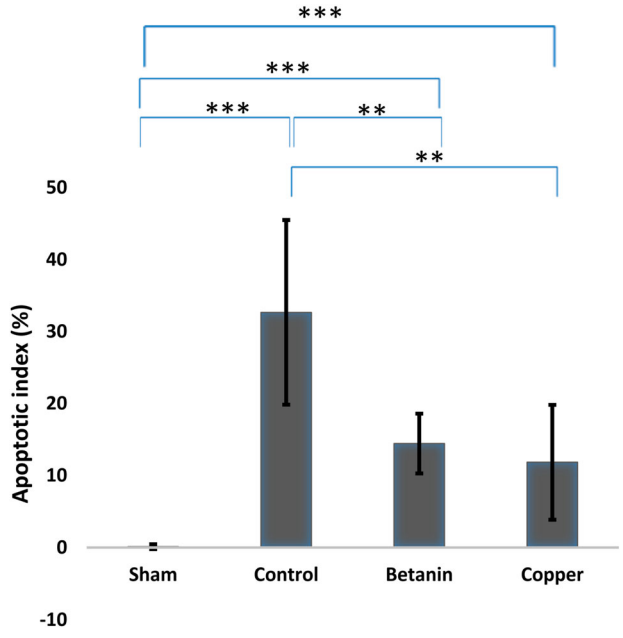

Figure 6 Apoptotic index for all groups in the spinal cord. Data represent means ± SD. Mann–Whitney *U* test. Control versus sham P = 0.001; betanin and copper groups versus control group P = 0.026 and P = 0.008, respectively. *n* = 7 for all groups. *P < 0.05, **P < 0.01 and ***P = 0.001.

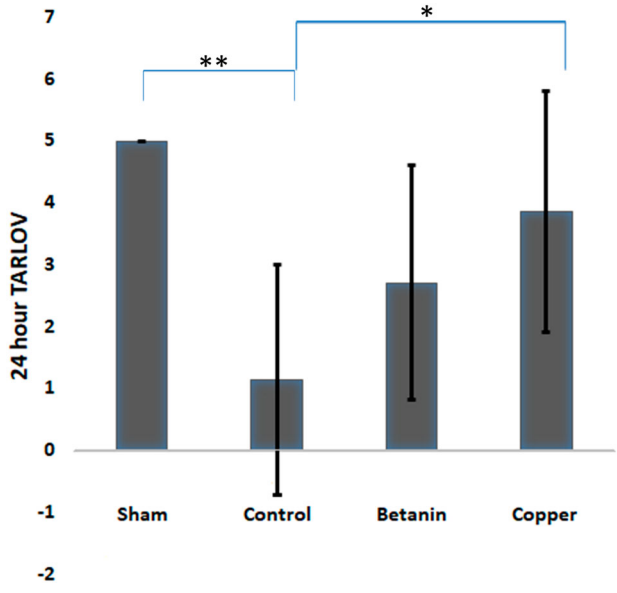

Figure 7 Tavlov scores in the 24th hour. Data represent means ± SD. Kruskal–Wallis test. Control versus sham P = 0.003; betanin and copper groups versus control group P = 0.076 and P = 0.026, respectively. *n* = 7 for all groups. *P < 0.05, **P < 0.01 and ***P = 0.001.

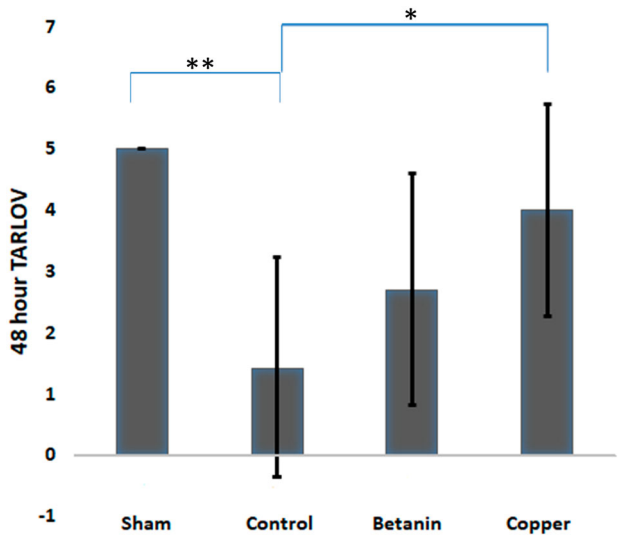

Figure 8 Tavlov scores in the 48th hour. Data represent means ± SD. Kruskal–Wallis test. Control versus sham P = 0.003; betanin and copper groups versus control group P = 0.190 and P = 0.038, respectively. *n* = 7 for all groups. *P < 0.05, **P < 0.01 and ***P = 0.001.

actual clinic scenario that results in this devastating complication.[19]

Antioxidant agents in amelioration of end-organ I/R injury have been studied in various experimental and clinical settings with favorable results[15,20] Among those studied, betanin has shown to be an avid electron donor and ROS scavenger. For example, in paraquat-induced renal injury in rats (meditated by induction of ROS during redox cycling), betanin showed broad-spectrum antioxidant capabilities, decreasing the amount of lipid peroxidation, modulating the inflammatory response and having a renal protective role. Copper has been shown to interfere with MPO activity *in vitro* and also was observed to get depleted soon after ischemic insult in a rat model of myocardial infarction. As copper is required for Hypoxia Inducible Factor 1 transcription and interaction, the researchers indicate that copper supplementation may ameliorate ROS injury in myocardium in an ischemia setting.[21] Copper is also shown to be depleted after formaldehyde inhalation injury in newborn rats, highlighting its importance as a coenzyme for SOD.[22]

In the present study, both betanin and copper sulphate were shown to ameliorate the markers of I/R injury in the spinal cord, namely, MDA level, MPO activity, GSH levels and apoptosis. Discrepancies were seen regarding of SOD activity and GSH levels, where copper sulphate over-performed betanin in spinal cord tissue. This fact may be due to the specific role of copper in SOD metabolism in neural tissue.[23,24]

In our study, decreased MDA levels and MPO activity in both treatment groups and increased SOD activity in copper group show that antioxidant properties of those materials are effective in spinal cord tissue. However, since copper is a cofactor of SOD enzyme, copper has a unique mechanism to increase SOD activity in CNS, where betanin failed to show any specific effect.

We observed that copper and betaine significantly decreased apoptosis when compared with the control group, but in the copper group, the apoptotic index decreased significantly closer to the sham group and copper over-performed betanin for this parameter. Even though both agents prevented apoptosis, only copper had an observable clinical effect, as shown in the Tarlov scores. Consistent with our study, previous studies have suggested that spinal cord tissue damage may have neurological deficits due to copper deficiency and that copper replacement may ameliorate neurological damage.[25,26] Betanine was widely investigated for antiapoptotic effects in a wide variety of organ systems except CNS,[27–30] our data also showed good anti-apoptotic effect but we failed to replicate this outcome clinically via Tarlov scores. As I/R injury has a variety of pathways[30,31] a multi-pronged approach with combination agents might need to be developed for better clinical outcome performance as apoptosis as a sole outcome did not seem to correlate with clinical function.

This study has several limitations. Due to the paucity of literature delineating neuroprotective effects and mechanisms of betanin and copper we did not plan for a combination treatment group initially. Also, the time point (48 h) was chosen as established in this surgical model.[15,32,33] Combination treatment with different carriers and usage of less debilitating models for more time intervals for sampling may be good approaches to extrapolate from our study in the future.

Conclusion

Betanin and copper have valuable cytoprotective roles in the spinal cord against I/R injury. Multiple pathways are probably involved in protective actions of betanin and copper sulphate, covering the spectrum of antioxidative, anti-inflammatory and anti-apoptotic properties. Further studies are needed for better delineation of action mechanisms.

Disclaimer statements

Contributors K.T. and E.K. conceived and designed the study; K.T., O.O., C.S.E. and O.M. contributed to the acquisition of data; K.T., O.O., Z.B. and I.T. contributed to the analysis and interpretation of data; K.T., O.O., Z.B., E.K., O.M. and C.S.E. drafted the manuscript; K.T., O.O., IT. and Z.B provided critical revision.

K.T., O.O., Z.B., E.K., O.M., C.S.E. and I.T gave final approval of the version.

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Conflict of interest Authors have no conflict of interest.

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