Silybum marianum reduces acute kidneys injury by modifying biochemical changes and oxidative stress levels in glycerol-induced CRUSH syndrome

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Crush syndrome (CS), also known as traumatic rhabdomyolysis, is a condition that can occur as a result of various incidents such as traffic accidents, earthquakes, and long-term crushing of muscle-rich parts. This condition arises due to the destruction of striated muscle cells. When the compressive pressure is suddenly removed, the damaged cells and myoglobin (Mb), which is nephrotoxic, are carried by oxygenated blood, leading to myoglobinuria, acute kidney injury (AKI), metabolic disorders, hypovolemic shock, and multiple organ dysfunction syndrome (MODS). This study aims to investigate the protective effects of Silybum marianum (S. marianum, SM) against induced toxicity resulting from glycerol (Gly) intramuscular injection (50 % glycerol; 0.9 % saline), which can lead to CS. The study involved 24 rats, which were randomly divided into four groups: (1) controls; (2) S. marianum treated (oral, 5 g/1 kg per day, 18 days); (3) Gly (8 mg/kg b.wt.: 50 % saline, intramuscular (i.m.), once, only on day 16); and (4) S. marianum (oral, 5 g/1 kg per day, 18 days) administered for 18 days, once per day, and Gly (8 mg/kg body weight: 50 % saline, i.m., once, only on day 16). By the end of the 19 experimental days of Gly administration, no mortality was observed in rats. After euthanasia, histopathological, biochemical and oxidative stress studies were performed on right kidney tissues and blood samples. All parameters of groups 1 and 2 were similar. The Gly-administered group showed significant weight loss (p < 0.003) compared to the control and S. marianum groups. On the other hand, the combined treatment Gly + S. marianum demonstrated a significant increase in antioxidant defense (p < 0.005). This can be attributed to the suppression of oxidative stress and reduced reactive oxygen/nitrogen production observed in the kidney and blood. Additionally, treatment with S. marianum provided protection against acute tubular necrosis, medullary congestion, and apoptotic indices recorded in the Gly group. Combination therapy of Gly and S. marianum improves tubular necrosis, activates antioxidant enzyme defense, and reduces free radicals. Longer treatment with this therapy can prevent CS (rhabdomyolysis).

Keywords: Crush syndrome, AKI, S.marianum, oxidative stress, protection

INTRODUCTION

Crush syndrome (CS) or traumatic rhabdomyolysis (RM) is a medical condition that results from direct injury to the body and is characterized by ischemic necrosis of muscle tissue due to prolonged limb compression or body swelling. This condition can cause electrolyte disturbance, dark urine (myoglobinuria), and elevated creatine kinase levels. These factors can lead to various clinical complications such as myoglobinuria, acute kidney injury (AKI), hypovolemic shock (HSc), and multiple organ dysfunction (MODS) [1, 2]. CS is a medical condition that frequently occurs in the aftermath of natural disasters like earthquakes, volcanic eruptions and traffic accidents, as well as during wars and stampedes. The syndrome is commonly seen in the aftermath of large-scale disasters that result in significant material losses and casualties. Although CS can affect all vital organs of the body, AKI is the most prominent complication.

AKI is a common consequence after CS, which leads to a decrease in renal function, electrolyte metabolism disturbances, and hypovolemic shock following the release of compression. The pathogenesis of CS-induced AKI is multifactorial and may involve renal ischemia-reperfusion (I/R) injury, systemic inflammation, and excessive deposition of myoglobin structures (Mb) in renal tubules released from damaged muscle tissue [2-4]. During CS, the breakdown of muscle cells leads

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to the release of large Mb amounts. After glomerular infiltration, Mb directly enters the renal tubules where it precipitates and promotes the formation of tubule-blocking casts. As a result, the damaged tubular epithelial cells lead to AKI. AKI clinically refers to an increase in serum creatinine by more than 0.3 mg/dl and a decrease in urine output by less than 0.5 mL/kg/h for six hours. Regrettably, even with the administration of dialysis or kidney transplantation, most CS individuals succumb to multiple organ failure due to a systemic inflammatory response [4, 5].

After the kidneys experience prolonged oxidative stress (OS), their blood flow is significantly reduced. Although the blood supply is restored after debridement, the subsequent I/R process triggers an accelerated production of reactive oxygen and nitrogen species (ROS/RNS), as well as an enhanced systematic inflammatory response [4]. Recent research has pointed towards ferroptosis (which involves iron retention, reduced glutathione (GSH), accumulation of ferro-dependent lipid peroxidation, and ROS) as a potential therapeutic target for reducing AKI following cardiac surgery [4, 6]. On the other hand, in renal tubules, Mb binds to uromodulin and uric acid, which promotes acute CS-AKI [7], and is phagocytosed by lysosomes to produce iron and Fe$^{2+}$ [8, 9]. Excessive filtration of Mb causes the activation of iron overload, which releases free divalent Fe$^{2+}$ ions. These ions produce hydroxyl radicals (•OH) via Fenton synthesis, leading to OS damage and lipid peroxidation in the affected cells. This can make it difficult to redox-modulate oxidative damage to the kidney [4, 10, 11]. In cases of AKI, ROS- and RNS-induced OS can cause significant damage to renal tubules, leading to prolonged lipid peroxidation and activation of cytoprotective mechanisms [12]. To counteract this, antioxidants such as vitamins E and C, N-acetylcysteine, dimethyl thiourea, melatonin, and selenium have been used in experimental animal models affected by glycerol-induced CS-AKI [12]. Additionally, natural plants have been found to protect kidney cells by reducing ROS and RNS levels, which are directly associated with oxidative stress in the kidneys. This helps modulate the oxidation of proteins, lipids, and nucleic acids while also restoring antioxidant enzyme inhibition [13].

The mechanism of action after CS-induced AKI and anti-inflammatory treatments highlight the importance of early intervention with antioxidant therapy to improve kidney function and treat AKI. This is crucial in preventing the progression of chronic nephropathy. The most commonly used method to induce CS/RM in rats and lead to AKI is through intramuscular injection of glycerol [14].

*Silybum marianum* L. (*S. marianum*) is a potent extract from milk thistle known for its anti-inflammatory, antioxidant, and AKI-protective properties. It is a cell permeability regulator and membrane stabilizer that boosts protein and nucleic acid synthesis in kidney cells, modulates immune-stimulatory cytokines and increases cell replication. *S. marianum* is effective in preventing premature death and is a must-have in any health professional’s arsenal [14, 18, 19]. This research aimed to evaluate the protective potential of *S. marianum* against Gly-induced AKI by assessing the performance of key antioxidant enzymes (GSH, SOD, and CAT), along with intracellular NO radicals and ROS production in the Wistar rats kidneys.

**EXPERIMENTAL**

**Gly-induced kidneys AKI and S. marianum co-treatment**

Twenty-four female Wister rats were housed in a controlled environment with a room temperature of 23°C and a 12-hour light/dark cycle. The rats (6 weeks old; 200-305 g), were kept at the Medical Faculty, Trakia University, Stara Zagora. The Research Ethics Committee/Medical Faculty (project code: MF7/2019; MF6/2022; BFSA 266/20), Trakia University and the European Directive 210/63/EU-22.09.2010 were strictly followed during the experiment. To prepare for the experiment, the female rats were deprived of water for 24 hours and then injected intramuscularly (i.m.) with a calculated dose of 50% v/v Gly/saline. The injection was divided equally into the right/ left hind limb (8 mg/kg body weight) and was given once on the 16th day of the experiment [20]. The experiment involved administering *S. marianum* (92% purity) orally at a concentration of 0.5 %, v/v (5 g/1 kg body weight) for 18 days, daily. Control animals were treated i.m. with physiological solution. After the treatment, each rat was allowed to recover for 3 days under laboratory conditions. On day 19, the rats were sacrificed under anesthesia using xylazine (270 mg/kg) and ketamine (30 mg/kg) administered i.p.

Blood (2 cm$^3$) was collected through cardiac puncture and centrifuged at 4000 rpm at 4 °C for 10 min and 200 µL of serum from each group was stored at -4°C until further use.

The kidneys were immediately collected, and washed with ice-cold saline. The left kidney from each animal was stored in 10% formalin for histological examination, while the right kidney was homogenized and, after the addition of solvents, centrifuged at 4000 rpm at 4 °C for 10 min.
Supernatants (300 µL) were prepared for biochemical analysis. The study was performed with 4 groups, 6 animals per group: (1) normal diet; (2) S. marianum treated (oral, 5 g/1 kg per day for 18 days); (3) Gly (8 mg/kg b.wt.: 50 % saline, i.m., once, only at day 16); and (4) S. marianum (oral, 5g/1 kg per day, 18 days) administered for 18 days, one per day and Gly (8 mg/kg b.wt.: 50 % saline, i.m., once, only at day 16th). By the end of the 19th day after Gly administration, no mortality was observed.

**Biochemical, enzyme-linked and oxidative stress markers evaluation**

Blood samples were centrifuged at 3000 rpm at 4 °C for 10 min until serum was separated, and blood urea nitrogen (BUN), creatinine (Cre), potassium (K+), and sodium (Na+) concentrations were determined spectrophotometrically (Sigma Aldrich, USA).

Kidney homogenates were centrifuged at 5000 rpm for 10 min and the supernatants were collected for the thiobarbituric acid- based method described by Ohkawa et al., 1979 [21], in μmole MDA/g tissue. Superoxide dismutase (SOD) and catalase (CAT) were measured using methods described by Sun et al., [22] and by Aebi et al., [23], respectively. The pro-inflammatory markers tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) were evaluated in serum using commercially available ELISA kits (Sigma Aldrich, USA) according to the manufacturer’s instructions. Kidney homogenates of 100 mg were homogenized with 900 μL 50 mM spin-trap N-tert-butyl-alpha-phenylnitrone (PBN) dissolved in dimethyl sulfoxide (DMSO) using one-cycle sonication (2 min). After 5 min of ice incubation, the suspension was centrifuged at 4000 rpm for 10 min at 4 °C, transferred into a cold Eppendorf tube and immediately analyzed by EPR spectroscopy [24] for ROS production. NO• radicals were studied by adapted EPR estimation of the spin-adduct formed between carboxy-PTIO K and generated radicals, by [25, 26].

**Histopathological examination**

Left kidneys were washed with saline, fixed in 10 % neutral formalin for 24 h, dehydrated in a series of increasing alcohol concentrations, and deparaffinized. Then, the sections were rehydrated and stained with hematoxylin/ eosin to quantify the extent of tubular injury, dilatation, vacuolization, and necrosis in kidney tissues [27].

**Statistical analysis**

Statistical analysis was performed with Statistica 8.0, Stasoft, Inc., one-way ANOVA, using Student’s t-test and Tukey-Kramer post hoc tests, to determine significant differences among data groups. EPR spectral processing was performed using Bruker Win-EPR and Sinfonia software. The results were expressed as means ± standard error of mean (SEM, n=6). p-Values less than 0.05 were considered as statistically significant.

**RESULTS AND DISCUSSION**

AKI due to CS is a serious condition that can lead to a loss of renal filtration rate and accumulation of protein residues in renal tissues. The Gly-induced CS-AKI model is used in animal research to understand the mechanisms causing this injury. This model displays a myoglobinuric state and a significant decrease in filtration rate, which is caused by the accumulation of ROS/ RNS resulting in inflammation [1, 2, 14]. Understanding AKI’s pathophysiology is crucial to develop effective treatments.

Plant antioxidants act as protectors and therapeutic agents against Mb-induced OS, preventing lipid peroxidation and ameliorating inflammatory response, ultimately preventing AKI damage. These findings demonstrate the potential for developing new treatments for this condition [4, 14, 28]. The study reported that administration of S. marianum significantly inhibited blood and renal inflammation in an AKI model/ferroptosis and reduced lipid peroxidation, OS and ROS/RNS formations.

*S. marianum* regulates biochemical parameters, electrolytes and lipid peroxidation in *CS*-induced AKI

The Gly-induction caused a significant increase in serum Cre (p < 0.05), BUN (p < 0.004), K+ (p < 0.05) in the AKI group compared to controls, indicating acute renal dysfunction associated with toxic renal Mb levels. In contrast, significant decrease in Na+ concentration in controls was not observed in the AKI group (Fig. 1).

In addition, *S. marianum* was significantly ameliorating Gly-induced AKI and directly regulating BUN, in comparison to animals receiving Gly alone. Interestingly, *S. marianum* administration in Gly-induced AKI animals insignificantly decreased Cre, K+, and Na+, but effectively ameliorated the renal injury (seen by histopathology). It significantly decreased lipid peroxidation levels to values almost comparable to healthy controls (300.9 ± 1.12 μmol vs. 274.4 ± 2.66 μmol, respectively), (Fig. 2).
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Figure 1. Effects of *S. marianum* and combination on biochemical parameters represented as mean ± SEM (n=6) for each group. To define difference per groups have used the Student t-tests; *p < 0.05 vs. the control group; **p < 0.05 vs. the Gly group.

Figure 2. Effects of *S. marianum* and combination on MDA levels in kidneys homogenates To define difference per groups have used the Student t-tests (n=6); *p < 0.05 vs. the control group; **p < 0.05 vs. the Gly group.

Administration of *S. marianum* (0.5 %) after Gly-induced AKI can alleviate signs of AKI, normalize BUN, and reduce renal edema. In addition, the administration of *S. marianum* lowers OS and reduces the accumulation of tissue lipids, which is probably due to the normalization of the relative gene expression of antioxidant enzymes [4, 14]. The presented results are in agreement with the studies of other researchers, according to which pretreatment with *S. marianum* reduces lipid peroxidation and lowers Gly-induced AKI, decreased myoglobinuric nephrotoxicity and renal ischemia [14, 29]. On the other side, at the cellular level, *S. marianum*, 0.5% administrated, is not sufficient to completely normalize the Gly-induced elevation in electrolytes (K⁺, Na⁺), biochemical (Cre) concentrations and oxidative changes, i.e. additional stimulation is needed to restore normal blood cells in the renal tubules with minimal protein deposition.

*S. marianum* regulates endogenous antioxidants, ROS production and nitric oxide (•NO) in CS-induced AKI

In CS-AKI rats, the heme and free ferro accumulation in the cytoplasm and mitochondria of kidney cells is remarkably increased, which causes a ferro-dependent lipid peroxidation and ROS/RNS increasement [30]. In addition, Liu et al., [31] found increased concentrations of hydrogen peroxide (H₂O₂), myeloperoxidase, and nitric oxide (•NO) in the serum and muscle of rats with experimentally induced CS-AKI. Moreover, CS-AKI is due to OS and free-radical processes, and treatment with antioxidants or radical scavengers suggests a beneficial therapeutic effect (Fig. 3).

Gly-induction resulted in a significant decrease in SOD and CAT activity (p<0.05; Figs. 3A, 3B) and a significant increase in ROS production and •NO concentration in the kidney (p < 0.003; Figs. 3C, 3D), compared to controls. Treatment with 0.5 % *S. marianum* significantly ameliorated Gly-induced AKI, restored renal endogenous enzymes, and protectively reduced renal OS by modulating ROS•NO concentrations compared to the Gly group (p<0.001).
Recent studies have reported that antioxidant molecules are able to protect against Mb-induced OS through ROS/RNS scavenging mechanisms, endogenous enzyme restoration, and alleviation of acute mitochondrial ROS production/lipid peroxidation in renal tubular cells [16-18]. *S. marianum*, 0.5 % may reduce ROS/RNS, following activation of the reperfusion injury salvage kinase (RISK) pathway, by inducing AKI/endothelial NO synthase (eNOS) activation and subsequent reduction of •NO radical mediated cytoprotective signaling [32, 33]. Reduced ROS/RNS generation suppresses the pro-inflammatory response and leads to full recovery of Gly-induced AKI, thereby reducing renal damage and increasing survival rate [32, 33]. In response to OS, proinflammatory...
cytokines, IL-6 and TNF-α, are released, activating macrophages and T-lymphocytes at the site of inflammation. Released IL-6, TNF-α, and c-Jun N-terminal kinase (JNK) are directly involved in the pathophysiological progression of CS-induced AKI [14, 34]. Rats exposed to Gly treatment revealed a significant increase in renal IL-6 and TNF-α circulation, compared to control (p < 0.05) (Figs. 4A, 4B).

In conclusion, this work presents S. marianum (0.5 %) as a stable antioxidant that can be potentially used to ameliorate the devastating Gly-induced signs of acute kidney injuries (remodeling the severe degenerative changes in renal corpuscles; accumulation of protein casts in the mesangial tissue; degeneration of renal tubules; severe congestion of the renal blood vessels) (Table 1), normalized BUN and alleviated renal OS and pro-inflammation, indirectly by preventing fibrotic processes, by long lasting Crush syndrome.

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