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Naringin attenuates cardiac and renal oxidative-inflammatory alterations in mice exposed to posttraumatic stress-induced alcohol use disorders

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ABSTRACT

Background: Increased oxidative stress and inflammation have important implications in cardiac and renal dysfunction associated with traumatic stress-induced alcohol use disorders (AUDs). However, little or no reports exist in literature on the cardiorenal impact of naringin, a bioactive natural flavonoid that is extracted from citrus plants with a plethora of pharmacological benefits in mice with single prolonged stress (SPS)-induced post-traumatic stress disorder (PTSD) and AUD-like conditions. Hence, this study investigated the effect of naringin on mice exposed to PTSD-AUD-induced cardiac and renal oxidative-inflammatory disturbances.

Methods: Mice were randomly assigned to six groups: group 1 received saline (10 mL/kg) as a normal control; group 2 received saline + SPS; group 3 received ethanol (20%, 10 mL/kg, or 2 g/kg); group 4 received SPS + ethanol (2 g/kg); group 5 received SPS + ethanol (2 g/kg) + Naringin (50 mg/kg, p.o.); and group 6 received SPS + ethanol (2 g/kg) + fluoxetine (10 mg/kg). Groups 3-6 received ethanol (20%, 10 mL/kg, equivalent to 2 g/kg) on days 8, 10, 12, 14, 16, 18, 20, in addition to concurrent medication treatments between PTSD days 8 and 21 to induce AUD.

Results: Our results showed that alcohol exacerbated PTSD-induced inflammatory response and cardiorenal oxidative stress, as evidenced by elevated heart and kidney levels of nitrites, malondialdehyde, tumor necrosis factor-alpha, and interleukin-6 and decreased glutathione, superoxide dismutase and catalase activities.

Conclusion: Naringin attenuates cardiac and renal oxidative-inflammatory alterations in mice co-exposed to posttraumatic stress-induced alcohol use disorders through inhibition of oxidative stress and release of pro-inflammatory cytokines.

INTRODUCTION

Post-traumatic stress disorder (PTSD) is a serious mental health disorder that people may develop directly or indirectly after experiencing a traumatic event. According to the DSM-5, symptoms of PTSD include flashbacks, avoidance behavior of the traumatic event or events, negative mood, and hyperarousal[1]. Veterans and military people exposed to battle have a higher risk of developing PTSD than civilians[2] and the lifetime prevalence of PTSD in the US population is estimated to be 6.8%[3]. Saraiya et al.[4] found that people with PTSD diagnosis have a 28–

85% increased risk of alcohol use disorder (AUD). When abstaining from alcohol, people with AUD are more likely to experience withdrawal symptoms, such as anhedonia and have a high tolerance to the short-term effects of alcohol[5]. Being an effective anxiolytic, alcohol is often used by people with PTSD as a self-medication to lessen their negative emotions and PTSD symptoms. Additionally, this may result in the emergence of AUD[6]. Individuals with comorbid PTSD and AUD have more intense alcohol cravings and relapse more frequently during withdrawal than those with AUD alone[7], suggesting that the comorbid disorder is different from either condition alone.

Alcohol has been demonstrated to boost the heart's ionotropic and chronotropic actions, which raises workload[8]. Cardiovascular issues linked to prolonged heart stimulation include myocardial infarction, hypertrophy and hypertension[9]. Importantly, reactive oxygen species are believed to damage lipid membranes and deplete antioxidants, ultimately resulting in inflammatory reactions[9]. These species are produced by the increased oxidative activities of mitochondria[9]. The intricate roles of the kidney and heart have enabled a functional network that supports the homeostatic process of blood pressure and fluid regulation [10]. However, the kidney's tubulo-glomerular functions have been limited by acetaldehyde, a product of alcohol metabolism[11,12].

There has been a scarcity of reports in the literature on the pharmaceutical interventions that can successfully mitigate the comorbidity between AUD and PTSD. Meanwhile, previous studies have reported that naturally occurring multi-targeted nutraceuticals possess therapeutic effects against stress-related disorders[13,14]. Naringin (4,5,7-trihydroxyflavanone-7-rhamnoglucoside), a bioactive natural flavonoid that is extracted from citrus plants, has neuroprotective, anti-inflammatory, anti-apoptotic, hepatoprotective, anticancer, antiulcer, antimicrobial, and antidiabetic pharmacological properties[15-21]. According to studies, naringin can help prevent or treat a variety of illnesses, including heart disease, diabetes, obesity and metabolic syndrome[22]. Naringin reduces damage from anticancer drugs, such as cyclophosphamide and cisplatin, to the kidneys, liver, and brain of rats by managing oxidative stress, inflammation, apoptosis, and autophagy[17,23]. The effects of naringin on inflammatory responses and cardiorenal oxidative disturbances brought on by alcohol consumption and a single, prolonged stressor have not, as far as we know, been studied. Therefore, the present study set out to investigate whether naringin could provide cardiorenal protection in mice that were exposed to posttraumatic stress-induced alcohol use disorders in mice.

MATERIALS AND METHODS

Animal and chemical utilities

All of the antioxidant reagents, fluoxetine (FLU), and naringin were purchased from Sigma-Aldrich in St. Louis, USA and Burgoyne Burbidges & Co. in Mumbai, India, respectively. Among them are potassium carbonate (Cat No. BDH9256), sodium chloride (Cat No. BDH9286), sodium carbonate (Cat No. BDH92284), sodium hydrogen phosphate (Cat No. BDH9298), formaldehyde (Cat No. 10790-708), and 5,5'-Dithiobis-2-nitrobenzoic acid (Cat No. D8130). Inflammatory cytokines examined using the BioLegend ELISA MAXTM Deluxe kit include TNF-α (CAT no. 430004) and IL-6 (CAT no. 431304). The ethanol (EtOH) was purchased from James Burrough Limited, a commercial supplier, in Nigeria. In this experiment, a variety of chemicals with higher purity levels were used. The animals utilized in this investigation were acquired and kept at Delta State University's Central Animal House in Abraka prior to the start of the trial. This study used female Swiss albino mice, weighing between 25 and 30 g and aged between 10 and 12 weeks. The mice were housed in a standard laboratory environment with a 12-hour cycle of equal light and dark, and they were given unlimited access to food and water before the experiment. The research was conducted following approval by the University's Ethics Committee (REC/FBMS/DELSU/23/187) and in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals (Volume 25, No. 28, revised in 1996), which were strictly adhered to.

Induction of single-prolonged stress

A single prolonged stress (SPS) is a multimodal traumatic stress induction paradigm that involves sequential exposure to three invasive stressors[24,25]. The mice's bodies and heads were restrained, with the pointed end covered by wire gauze to permit airflow, while they were kept in a 50 mL Falcon tube (a restraint stress device) for 2 h. Additionally, the mice were made to swim against their will for 20 min inside a plexiglass cylinder measuring 50 cm high and 24 cm in diameter, filled two-thirds with water at 24 °C. After drying the mice and giving them another 15 min to recover, they were exposed again to pure diethyl ether vapor until they lost consciousness. To induce PTSD, the mice were then immediately placed in separate cages and left alone for seven days[26,27].

Alcohol use disorder induction

After PTSD induction, AUD was induced by gavage with 20% v/v, or 2 g/kg of EtOH, using a well-known every-other-day access strategy[28,29]. This EtOH dosage and injection strategy markedly increased the severity of the SPS-PTSD-like syndrome. It showed strong signs of dependence, which were previously

observed in the first study[30,31]. During the 14 days after SPS induction, the mice in groups 3 through 6 were administered 2 g/kg of EtOH every other day on days 8, 10, 12, 14, 16, 18, and 20. They also received naringin and FLU treatments concurrently from PTSD days 8 to 21.

Experimental design

Following 7-days of acclimatization, mice were randomly assigned to six groups: group 1 received saline (10 mL/kg) as a normal control; group 2 received saline + SPS; group 3 received EtOH (20%, 10 mL/kg, or 2 g/kg); group 4 received SPS + EtOH (2 g/kg); group 5 received SPS + EtOH (2 g/kg) + Naringin (50 mg/kg, p.o.); and group 6 received SPS + EtOH (2 g/kg) + FLU (10 mg/kg). Groups 3–6 received EtOH (20%, 10 mL/kg, equivalent to 2 g/kg) every other day (that is, on days 8, 10, 12, 14, 16, 18, 20) in addition to concurrent medication treatments between PTSD days 8 and 21 to induce AUD, which is believed to exacerbate PTSD-like symptoms [31]. Thus, depending on the groups, freshly prepared naringin and FLU were administered once daily for 14 consecutive days, from day 8 to day 21. However, sterile saline (10 mL/kg/p.o./day) was administered to the mice in groups 1 (saline control) and 2 (SPS control) for 14 days. Pre-dissolved in saline, the doses of FLU (10 mg/kg) [27], naringin (50 mg/kg) [31, 32] and a vehicle (VEH) (saline, 10 mL/kg) were selected from the findings of previous studies and administered orally (p.o.). Following the exposures, mice were cervically dislocated and euthanized on day 22. The kidney and heart were isolated and weighed using a sensitive weighing scale, homogenized, and centrifuged in a cold (4 °C) centrifuge (Anke TGL-16 G, Nanjing, China) for 10 min at 10,000 rpm. The supernatant was gathered for biological analysis.

Glutathione (GSH) level measurement

To measure the amount of glutathione in the cardiac and kidney tissues, Ellman used the process of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) forming a yellow complex[33,34]. The yellow mixture that developed at 412 nm was measured using a spectrophotometer (UV-1650 PC, Shimadzu, Japan) after DTNB (0.04%) was added to the supernatant and allowed to sit at room temperature for 20 min. The results were then displayed as tissue moles with micromoles per milligram protein.

The malondialdehyde (MDA) assay

Lipid peroxidation in the in the cardiac and kidney tissues was measured by measuring the amounts of malondialdehyde (MDA). Using this method, homogenized tissue was mixed with 10% w/v TCA in an acidic environment, and the samples were centrifuged at 3000 g for 10 min. The supernatant was cooked in a boiling water bath for an hour after adding TBA solution (0.67%, w/v) to give it a pink tint. After cooling, the absorbance at 532 nm was measured using a spectrophotometer (UV-1650 PC, Shimadzu, Japan). They calculated the amount of MDA and represented it as mol/g tissue using the molar extinction coefficient of E¼1.56_105/M/cm[35,36].

Nitrite measurement

The nitrite concentration was determined in the cardiac and kidney tissues using the Griess reaction technique to assess the NO concentration in the samples. The color changes were measured using a spectrophotometer set to 540 nm[37,38].

Catalase activity

Catalase (CAT) activity in the cardiac and kidney tissues was assessed using a modified L. Goth approach[39]. H_2O_2 (10 mM) was added to a supernatant (obtained by centrifuging homogenized tissue at 12,000 g for 20 min at 4 °C) in Tris-HCl (0.05 mmol) and left to stand at room temperature for 10 min. After 4% ammonium molybdate was added, the absorbance at 410 nm was measured. The result was expressed as U/mg protein.

Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was assessed in the cardiac and kidney tissues according to the kit's guidelines (Randox Labs, Crumlin, UK). SOD accelerates the production of the toxic superoxide radical (O_2^-) from oxidative stress mechanisms, converting it into molecular oxygen and hydrogen peroxide. This method yields red formazan dye by reacting 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) with superoxide radicals generated by xanthine and xanthine oxidase (XOD). The SOD activity in this process is then determined by the degree of inhibition. According to the test conditions, one unit of SOD inhibits the rate of decrease in INT by 50%[40]. The result was expressed as U/mg protein.

Glutathione S-transferase (GST) activity

An activity test for glutathione s-transferase (GST) was performed in the cardiac and kidney tissues using a GST kit (Randox Labs, Crumlin, UK). GST promotes the oxidation of glutathione by cumene hydroperoxide. Glutathione reductase and NADPH cause GSSG to be immediately reduced while simultaneously oxidizing NADPH to NADP. It was discovered that the absorbance was lower at 340 nm[41].

Tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)

According to the manufacturer's instructions, the levels of TNF-α and IL-6 in the supernatant of mice from cardiac and kidney tissues were measured using an ELISA kit (BioAssay Systems, Hayward, CA, USA). Every sample, reference solution, and reagent was allowed to reach room temperature before use. The samples, standards, controls, and streptavidin solution were placed on a microplate and coated with a biotinylated antibody. The microplate was then allowed to sit at room temperature for 45 min. We supplied 100 µL of TMB One-Step substrate to each well and let it settle at room temperature for half an hour before adding the stop solution (50 µL). A log-log logistic curve-fit was used to determine the unknown sample concentrations in pg/mL following a reading at 450 nm using a Spectramax M-5 (Molecular Devices, Sunnyvale, CA) multifunctional plate reader equipped with a Softmax Pro v 5.4 (SMP 5.4).

Statistics

The data were shown as mean ± SEM for six animals per group. The statistical analysis was completed using Tukey's post hoc test and the one-way analysis of variance (ANOVA) test with GraphPad Prism 5.0 (San Diego, CA, USA). Statistical significance was defined as a p-value of less than 0.05.

RESULTS

Naringin suppressed the actions of cardiorenal pro-oxidants in mice exposed to PTSD-AUD

Mice exposed to intermittent alcohol consumption as well as SPS significantly resulted in increased activities of pro-oxidant-like molecules such as heart MDA [F (5, 18) = 14.78, P<0.0001, $R^2=0.8042$] and nitrite [F (5, 18) = 34.92, P<0.0001, $R^2=0.9065$] as well as kidney MDA [F (5, 18) = 29.58, P<0.0001, $R^2=0.8915$] and nitrite [F (5, 18) = 13.10, P<0.0001, $R^2=0.7845$] when compared with normal control mice (Figure 1A-D). However, therapy with naringin significantly suppressed the activities of these molecules, as evidenced by reduced cardiac and kidney levels of MDA and nitrite, which were comparable to those of mice treated with fluoxetine.

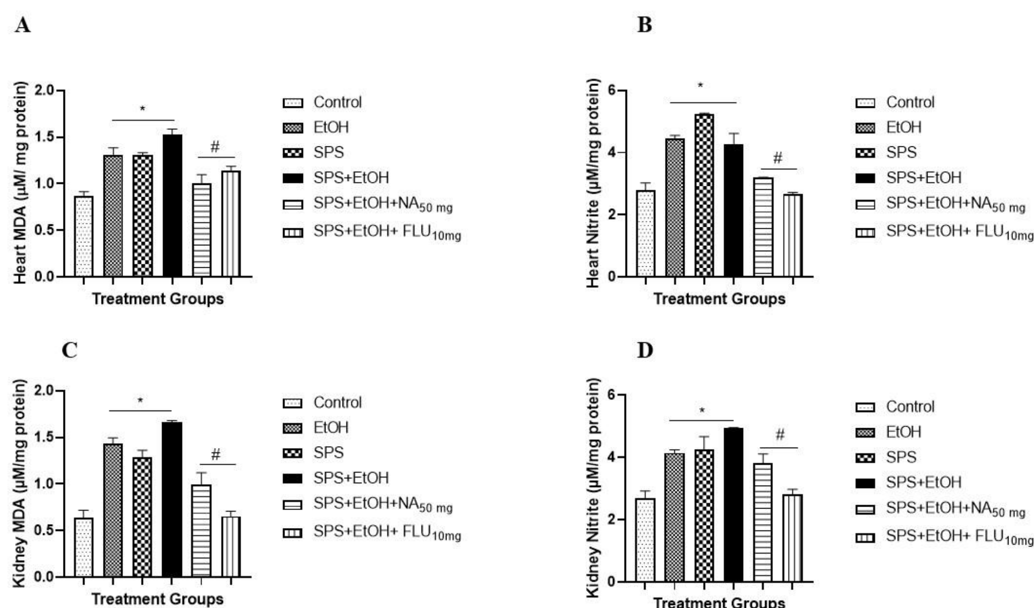

Figure 1: Effects of Naringin on the actions of cardiorenal pro-oxidants (A) Heart malondialdehyde (MDA), (B) Heart nitrite, (C) kidney malondialdehyde (MDA),(D) kidney Nitrite. Data were presented as mean ± SEM, n = 5, and $*p < 0.05$ was significant when compared with the normal control, and $\#p < 0.05$ was significant when compared with SPS + EtOH. EtOH = ethanol, SPS = single prolonged stress and FLU = Fluoxetine.

Cardiorenal *in vivo* antioxidant-enhancing properties of naringin in mice exposed to PTSD-AUD

Alcohol-mediated production of pro-oxidants, which were exacerbated by exposure to SPS resulted in significant decrease in cardiac catalase [F (5, 18) = 25.04, P<0.0001, $R^2=0.8743$], SOD [F (5, 18) = 93.63, P<0.0001, $R^2=0.9630$] and GSH [F (5, 18) = 20.55, P<0.0001, $R^2=0.8510$] as well as Kidney catalase [F (5, 18) = 57.52, P<0.0001, $R^2=0.9411$], SOD [F (5, 18) = 16.51, P<0.0001, $R^2=0.8210$] and GSH [F (5, 18) = 12.29, P<0.0001, $R^2=0.7735$] in

mice co-exposed with intermittent alcohol consumption and SPS when compared with normal control mice. However, the in vivo cardiorenal anti-oxidant armories were significantly enhanced following naringin therapy as evidenced in the significant increase in cardiac catalase, SOD and GSH as well as renal catalase, SOD and GSH when compared with co-exposed mice, which was similar to the observation in fluoxetine-treated mice (Figure 2A-F).

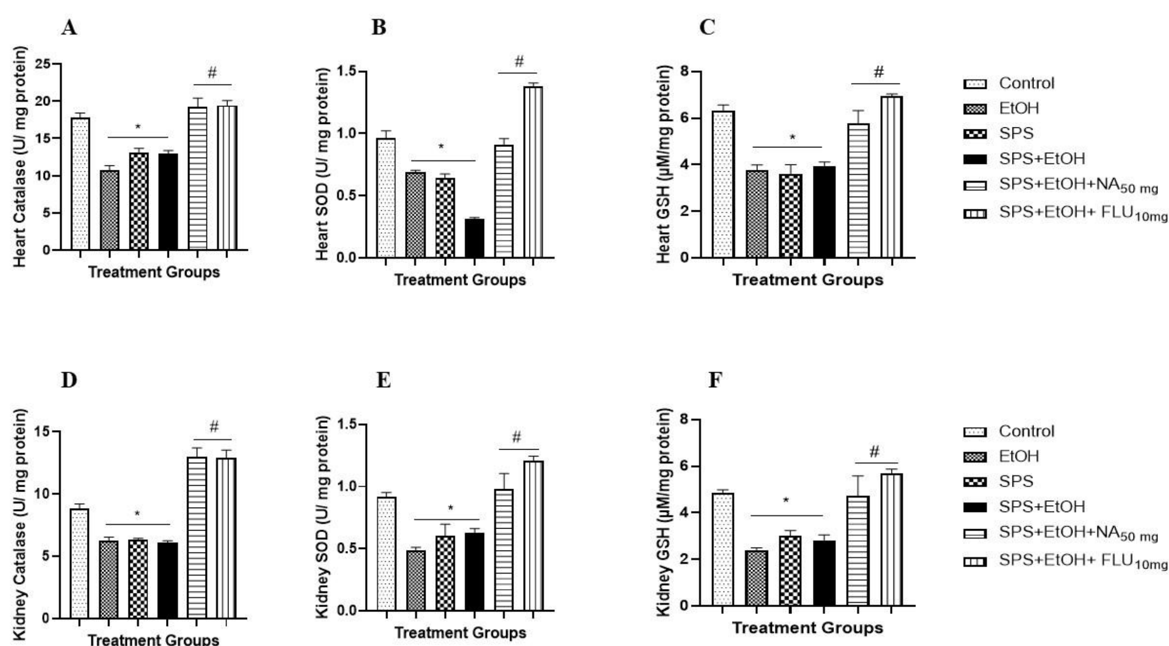

Figure 2: Cardiorenal *in vivo* antioxidant enhancing properties of naringin (A) Heart catalase, (B) Heart superoxide dismutase (SOD), (C) Heart glutathione (GSH), (D) Kidney catalase, (E) Kidney superoxide dismutase (SOD), (F) Kidney glutathione (GSH). Data were presented as mean ± SEM, n=5 and *$p<0.05$ was significant when compared with normal control, and #p < 0.05 was significant when compared with SPS+EtOH. EtOH = ethanol, SPS = single prolonged stress and FLU = Fluoxetine.

Effect of Naringin on cardiorenal pro-inflammatory responses in mice exposed to PTSD-AUD

Mice co-exposed to SPS and intermittent alcohol consumption had a significant increase in cardiac TNF- α [F (5, 18) = 29.43, P<0.0001, $R^2=0.8910$] and IL-6 [F (5, 18) = 36.92, P<0.0001, $R^2=0.9112$] as well as renal TNF-α [F (5, 18) = 50.26, P<0.0001, $R^2=0.9332$] and IL-6 [F (5, 18) = 43.53, P<0.0001, $R^2=0.9236$] when compared with normal control mice. Treatment with naringin inhibited the cardiorenal pro-inflammatory reactions by reducing cardiac TNF-α and IL-6 as well as renal TNF-α and IL-6 when compared with co-exposed mice (Figure 3A-D).

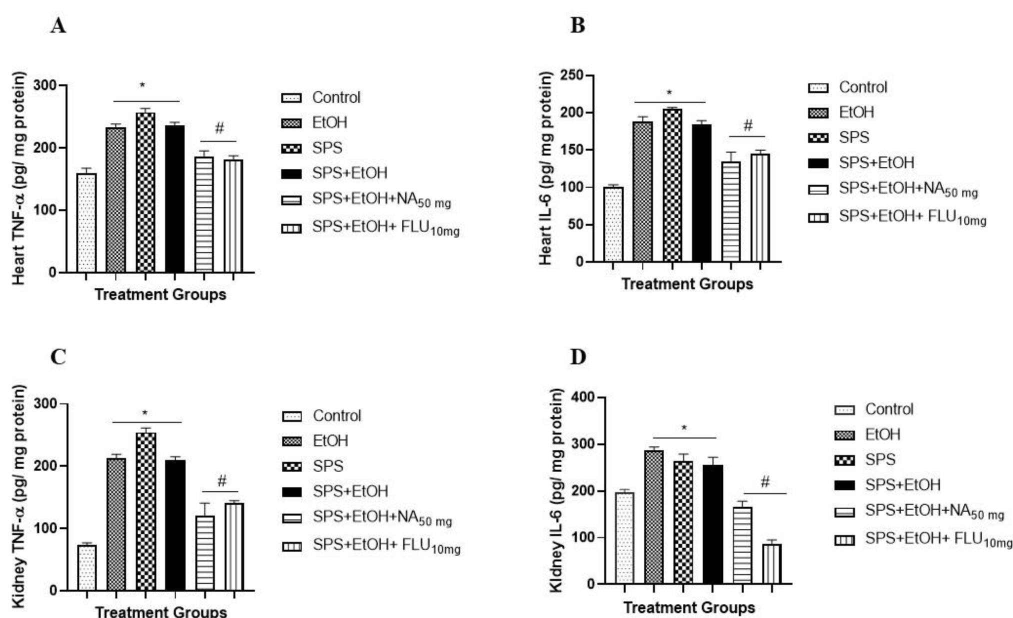

Figure 3: Naringin inhibits cardiorenal pro-inflammatory responses (A) Heart tumor necrosis factor alpha (TNF-α), (B) Heart interleukin-6 (IL-6), (C) Kidney tumor necrosis factor alpha (TNF-α), (D) Kidney interleukin-6 (IL-6). Data was presented as mean ± SEM, n=5 and *$p<0.05$* was significant when compared with normal control and #$p<0.05$ was significant when compared with SPS+EtOH. EtOH = ethanol, SPS = single prolonged stress and FLU = Fluoxetine.

DISCUSSION

Increased oxidative stress and inflammation play a significant role in cardiac and renal dysfunction related to traumatic stress-induced AUDs. From this study, mice subjected to both chronic intermittent alcohol consumption and SPS experienced renal and cardiac inflammation and oxidative stress, which ultimately resulted in poisoning. However, naringin administration protected the kidney and heart by lowering the production of free radicals, bolstering antioxidant defenses, and prevented the release of proinflammatory cytokines.

Lifestyle, dietary choices and genetic inclinations have all been found to have an impact on heart and kidney functions[42]. It has been demonstrated that the numerous stressors that humans encounter daily have a detrimental impact on a person's psychological, emotional and social well-being[43]. Several studies have shown that vulnerable persons resort to drinking as a coping strategy, resulting in the development of AUD[31,44]. This condition affects the kidneys and heart by activating several pathological signaling pathways, notably linked to increased oxidative stress and release of pro-inflammatory cytokines[44].

Additionally, the increased production of free radicals has been linked to the metabolic process of alcohol, which in turn alters the normal functioning of the heart[45]. It has also been observed that alcohol use accelerates the heart's metabolic activities, causing the release of toxic chemicals such as H_2O_2, NAD^+ and NADH[27,46]. This study suggests that cardiorenal dysfunction may be caused by increased oxidative/nitrergic stress, increased inflammatory activity and insufficient endothelial nitric oxide synthase (eNOS) signaling[27,47,48]. In mice co-exposed to EtOH and SPS, the MDA and nitrite levels in the cardiac tissues were markedly increased compared to the control. Glutamatergic signaling and the production of pro-inflammatory cytokines are both mediated by elevated nitrite levels, which ultimately lead to excitotoxicity[47,48,49]. Previous studies have shown that alcohol promotes protein oxidation and cardiorenal cell membrane lipid peroxidation by increasing the quantities of toxic metabolic products, such as acetaldehyde[50,51]. However, treatment with naringin significantly reduced the heart's nitrite and MDA levels, which clearly implies a reduction in lipid peroxidative diseases. The effects of fluoxetine were similar to this observation.

In the past, oxidative stress was defined as an imbalance between pro-oxidants and antioxidants[38,48]. Several diseases associated with AUD have been linked to increased metabolic product such as acetaldehyde, which is one of the first by-products of alcohol with pro-oxidant effect, notably involving decrease alcohol and aldehyde dehydrogenases, which have antioxidant action[52]. In vivo antioxidant armories are the first line of defense against pathological diseases caused by chemical substances, including alcohol. In this study, we found

a significant decrease of antioxidants, such as catalase, GST, SOD, and GSH in the heart and kidneys of mice co-exposed to EtOH and SPS, suggesting the presence of oxidative stress in the tissues. Antioxidants are primarily used to fight, hydrolyze, and detoxify toxic chemicals such as H_2O_2, NAD^+ and NADH. However, the depletion of antioxidants suggests that these cytotoxic chemicals have accumulated, leading to oxidative stress and disrupting the equilibrium[53]. Surprisingly, naringin therapy, similar to the effect of fluoxetine, significantly increased the levels of catalase, GST, SOD, and GSH in the kidneys and heart compared to the co-exposed group. These results are consistent with recent studies, which reported that naringin possesses antioxidant qualities in brain and testicular tissues, suggesting a decrease in cardiorenal oxidative abnormalities[54,55].

Various studies have established a connection between immune cell activation and oxidative damage[56,57,58]. When increased free radical production in the heart, driven by heightened metabolic activity from alcohol's stimulating effects, damages lipid membranes, it leads to the production of pro-inflammatory cytokines. In this study, both heart and kidney tissues showed notably higher levels of TNF-α and IL-6, suggesting that combined exposure to EtOH and SPS triggered an inflammatory response. Conversely, naringin treatment exhibits anti-inflammatory effects similar to fluoxetine, as it significantly reduces the release of these pro-inflammatory cytokines.

CONCLUSION

Alcohol exacerbated PTSD-induced inflammatory reactions and cardiorenal oxidative stress, as evidenced in elevated kidney levels of nitrites, MDA, TNF-α, and IL-6 and decreased GSH, SOD, and catalase activities.

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Declarations

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Availability of data and material

Available upon reasonable request

Code availability

Not applicable

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