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ORIGINAL ARTICLE

# The targets of β-sitosterol as a novel therapeutic against cardio-renal complications in acute renal ischemia/reperfusion damage

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## Abstract

This research is the first to use β-sitosterol on myocardial and renal tissues in renal ischemia/reperfusion (IR) damage. Female Wistar rats were randomly divided into three groups: control (sham), renal IR (50 min ischemia – 3 h reperfusion), and renal IR + 150 mg/kg/p.o. β-sitosterol (the rats were treated with β-sitosterol orally once 1 h before the IR procedure). β-Sitosterol pretreatment caused an increase in superoxide dismutase and glutathione activities and a decrease in malondialdehyde levels in the kidney and heart. Moreover, it alleviated histopathological changes and downregulated the levels of tumor necrosis factor-alpha and interleukin-6 and upregulated the levels of endothelial nitric oxide synthase. As conclusion, the potential of β-sitosterol for renal and cardiac necrosis and apoptosis appears to act by limiting inflammatory response and oxidative stress. Thus, the potential of this compound is noteworthy and may serve as a potential therapeutic in the treatment of acute organ damages due to renal IR.

**Keywords** Ischemia/reperfusion · Kidney · Heart · β-Sitosterol · Inflammation · Oxidative stress

## Introduction

Renal ischemia/reperfusion (IR) injury is an important cause of acute kidney failure and also remote organ damages. Despite progress in the therapeutic approach in the recent years, it still has high prevalence and mortality rate. Ischemia commonly occurs due to occlusion in blood vessels generated hypoxia condition in the kidney (Zheng et al. 2019). Reperfusion following ischemia as an important cause of renal pathophysiology targets the restoration of arteriovenous kidney (Ono et al. 2019). All these episodes enhance the production of free radicals in kidney cells and thereby the injury cascade (Kim et al. 2019). Dysfunction induced by free radicals in the ischemic-reperfusive injury is well documented with regard to vital organs such as brain, kidney, heart, and liver. There is evidence that the antioxidant strategies may attenuate organ dysfunctions by reducing the production of ROS. However, no effective protective therapy is currently used for heart and kidney injuries associated with IR.

The numerous bioactive phytosterols are able to provide health benefits. Recently, one major interest for proposing as dietary supplement β-sitosterol is that it has anti-hypercholesterolemic impacts. β-Sitosterol is also recommended as neuroprotective, antimutagenic, antidiabetic, antifungal, antibacterial, and chemoprotective agent (Kuo et al. 2019). Considering the presence of β-sitosterol in different pharmacological applications, its effects have been especially associated with reduced oxidative stress in diseases such as cardiovascular disorders, aging, and cancer (Brinton et al. 2018; Jiang et al. 2019). On the other hand, the nanoribbons, which contain β-sitosterol, can be utilized as a promising anti-inflammatory material in wound healing (Amina et al. 2017).

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The histological findings, which were the most reliable diagnostic criteria for organ damages (Nepal et al. 2012), and analysis of the reliable biomarkers might be the key in order to achieve a more critical evaluation of the obtained results (Lishchuk et al. 2019). β-Sitosterol could be a suggestive candidate for organs with increased vulnerability toward IR. Thus, the aim of this study was to measure the capability of β-sitosterol in attenuating renal IR injury, with special attention to the kidney and heart. With this aim, we performed the histological and functional studies in rat renal IR model. We were also framed to examine the antioxidant and anti-inflammatory effects of β-sitosterol biochemically.

## Material and methods

### Animals and surgical procedure

Experiments were conducted in female Wistar rats (adult, weighing on average 300 g) maintained on a standard chow pellet diet with tap water ad libitum. Animals were kept under standard laboratory conditions, maintained in temperature 22–25 °C and humidity-controlled rooms on a 12-h/12-h light/dark cycle. All animals were randomly assigned to three groups ($n = 6$ each): (I) sham-operated control; (II) renal IR; (III) renal IR + β-sitosterol 150 mg/kg/p.o. The dose of β-sitosterol selected according to the literature data was dissolved in distilled water and diluted at 150 mg/kg concentration (Gupta et al. 2011). β-Sitosterol was administered orally to the treatment group 1 h before the IR procedure, whereas the sham group received only vehicle (1% CMC solution). Rats were anesthetized with sevoflurane, abdominal incisions were made, and bilateral renal ischemia was induced by clamping renal pedicles for 50 min with nontraumatic microaneurysm clamps. After removing the clamps, kidneys were inspected for restoration of blood flow and two layers were sutured. Rats were sacrificed by an overdose of sevoflurane 3 h after reperfusion. Sham-operated animals were subjected to the same procedure, excluding the placement of the clamps. At the end of the experiment, the blood was collected for estimation of serum urea and creatinine, and the kidney and heart tissues were harvested for biochemistry and histopathology analyses. The tissues were rinsed with cold normal saline to remove blood contaminant and then immediately immersed in liquid nitrogen and stored at − 80 °C until further biochemical assessment. For histology assessment, the tissue samples were directly fixed with 10% buffered formalin phosphate. The values of serum urea and creatinine values were analyzed through an enzymatic method (Abbott Diagnostics Division, Abbott Laboratories Abbott Park, IL, USA).

### Assay of oxidative stress and inflammation

Kidney and heart tissues were homogenized with EBC buffer and then centrifuged for 10 min at 10,000 ×$g$ at 4 °C. After centrifugation, supernatant aliquots were assayed for superoxide dismutase (SOD), glutathione (GSH), malondialdehyde (MDA), endothelial nitric oxide synthase (eNOS), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) levels in the tissues using enzyme-linked immunosorbent assay (ELISA) kits (SunRed Bio., China).

### Histopathological and immunohistochemical assessments

The fixed heart and kidney tissues were dehydrated in graded ethanol solutions. Paraffin-embedded tissue samples were sectioned onto slides and processed for hematoxylin and eosin (H&E) and cleaved caspase-3 immunostaining. The sections were deparaffinized and applied diaminobenzidine (DAB) as chromogen; slides were counterstained with hematoxylin, dehydrated, and covered by coverslips. Immunohistochemical staining of cleaved caspase-3 was performed using anti-cleaved caspase-3 antibodies (Abcam, ab2302; 1:2500 dilution) with a Novolink Polymer Detection kit (Leica Microsystems Pte Ltd., Taipei, Taiwan), following the manufacturer's instructions. Analysis of the sections was performed by the same pathologist blindly using a light microscope (Leica DM 1000, Germany). The pathologists continuously observed at least 10 high-power fields (× 200) for each slice.

### Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM) for the experimental group. The comparisons were done by a one-way analysis of variance (ANOVA) test followed by GraphPad prism 5.0 statistics software (GraphPad, La Jolla, CA, USA). Tukey's test was used as a post hoc. A $p$ value of less than 0.05 was considered statistically significant.

## Results

### Effect of β-sitosterol on inflammation response in renal IR

As shown in Fig. 1, renal IR group showed a significant increase in the TNF-α and IL-6 levels ($p < 0.0001$) in tissues and a reduction in the eNOS level for both kidney and heart as compared with sham group rats ($p < 0.0001$). On the other hand, oral pretreatment with β-sitosterol 150 mg/kg/p.o. significantly decreased the TNF-α and IL-6 levels ($p < 0.0001$)

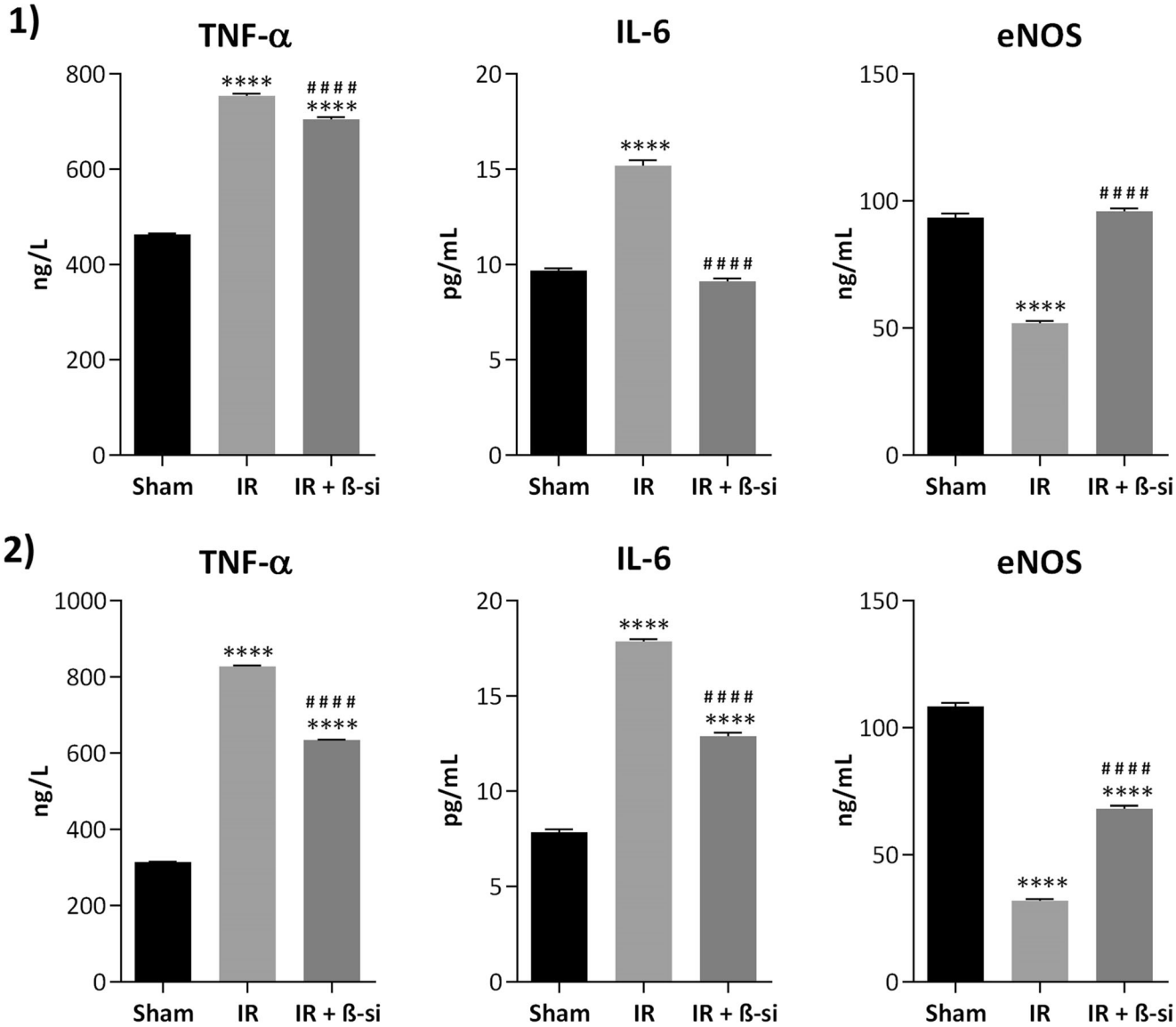



**Fig. 1** The effects of β-sitosterol and the TNF-α, IL-6, and eNOS levels on kidney and heart after renal IR injury. 1) Kidney. 2) Heart. Data are presented as mean ± SEM ($n = 6$). The asterisks denote significant differences between other studied groups and sham (****$p < 0.0001$). The number signs denote significant differences between other studied groups and I/R group ($^{\#\#\#\#}p < 0.0001$) by Tukey's multiple range tests. IR ischemia/reperfusion, β-si β-sitosterol

and increased the eNOS level as compared with the renal IR group ($p < 0.0001$).

## Effect of β-sitosterol on oxidative defense system in renal IR

As shown in Fig. 2, renal IR group showed a significant decrease in SOD and GSH levels ($p < 0.0001$) and increase in MDA level ($p < 0.0001$) in rat kidney and heart tissues as compared with sham group rats. In contrast, administration of β-sitosterol 150 mg/kg/p.o. resulted in significant increase in SOD and GSH levels ($p < 0.0001$) and reduction in MDA level ($p < 0.0001$) in rat kidney and heart tissues subjected to renal IR.

## Effect of β-sitosterol on apoptosis in renal IR

As shown in Fig. 3, cleaved caspase-3 immunoreactivities on kidney and heart tissues were markedly increased in the renal IR group as compared with sham group rats. However, reduced cleaved caspase-3 immunoreactivities were detected in the both tissues treated with β-sitosterol before renal IR.

## Effect of β-sitosterol on serum parameters and histopathological changes in with renal IR

As shown in Figs. 4a and 5a, histopathological examination by H&E staining revealed normal histology of kidney and heart in sham group. The renal IR caused severe hemorrhage,

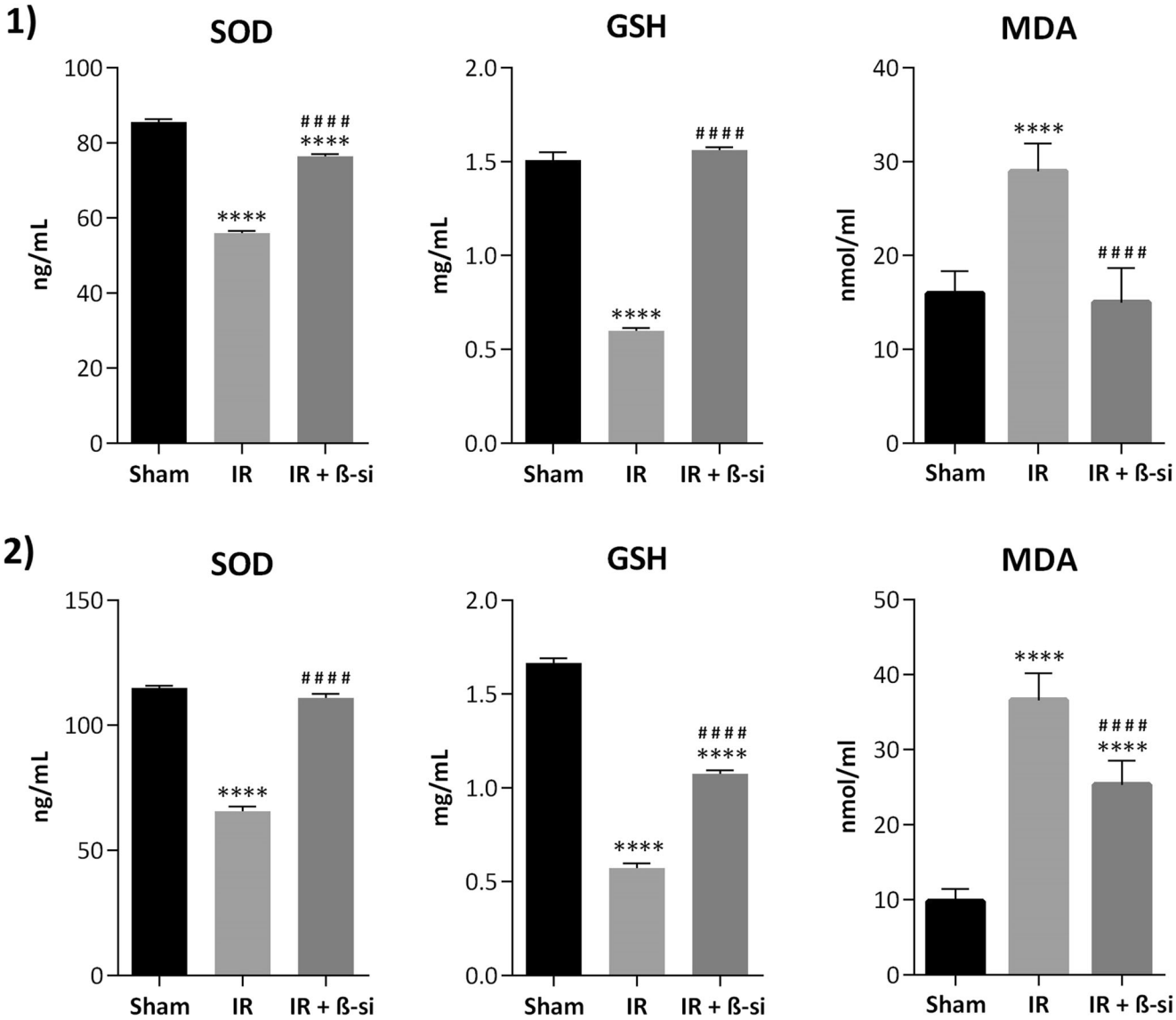
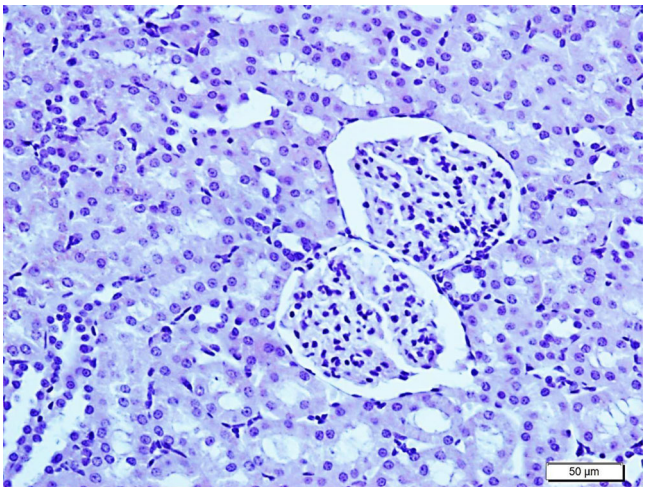



**Fig. 2** The effects of β-sitosterol and the SOD, GSH, and MDA levels on kidney and heart after renal IR injury. 1) Kidney. 2) Heart. Data are presented as mean ± SEM ($n = 6$). The asterisks denote significant differences between other studied groups and sham (****$p < 0.0001$). The number signs denote significant differences between other studied groups and renal IR group (####$p < 0.0001$) by Tukey's multiple range tests. IR ischemia/reperfusion, β-si β-sitosterol

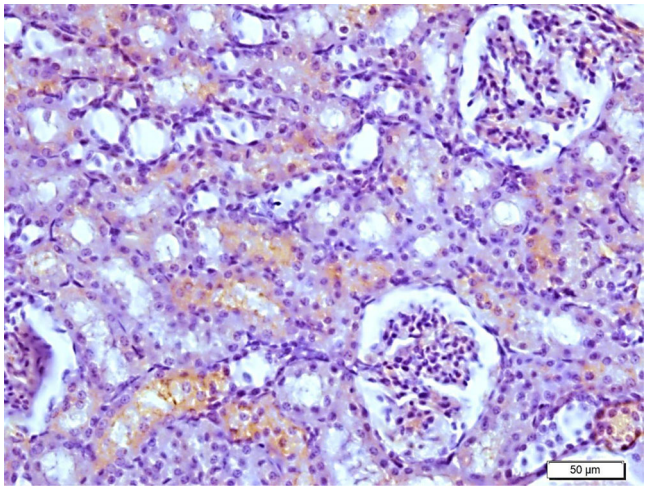
congestion, apoptosis, necrosis, tubular dilatation, and hyalinization in kidney tissue (Fig. 4b–e; Table 1). On the contrary, when the β-sitosterol 150 mg/kg/p.o. were applied on rat kidney before IR, the reduction in the number of these histopathological changes was observed, and tubular congestion was slightly observed (Fig. 4f). Moreover, compared with normal heart, renal IR induced a significant heart injury that is featured with remarkable congestion, hemorrhage, apoptosis, necrosis, and cell infiltration (Fig. 5b–d; Table 2). However, treatment with β-sitosterol 150 mg/kg/p.o. before renal IR significantly reduced heart injury as indicated by a reduction in the number of histopathological change (Fig. 5e). In addition, the levels of urea and creatinine significantly elevated in the renal IR group rats ($p < 0.0001$ and $p < 0.001$, respectively) while decreased in rats pretreated with β-sitosterol 150 mg/kg/p.o. ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 6).

## Discussion

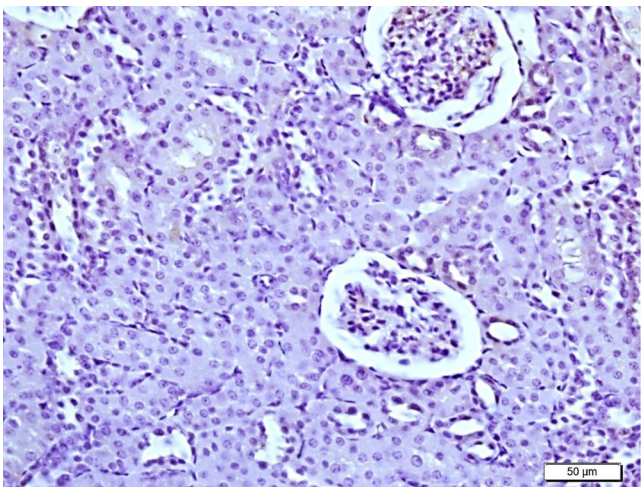
IR causes to generate impactful changes in kidney morphology and function (Zankar et al. 2019). In studies conducted in recent years, it is thought that there is a complex interaction between kidney and heart. The renal and cardiovascular alterations decrease survival rates. That is why more research is needed to develop the effective treatment agents and strategies (Neuen et al. 2018). In this study, we have evaluated for the




1) Kidney, **Sham**; Caspase-3 negative,

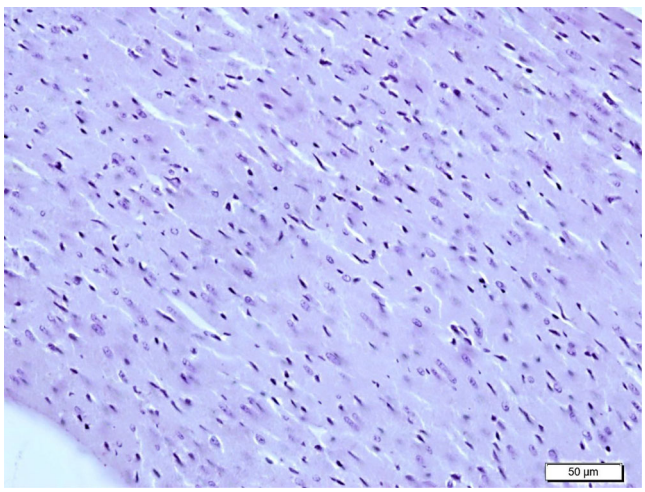



1) Kidney, **IR**; severe Caspase-3 immunoreactivity,

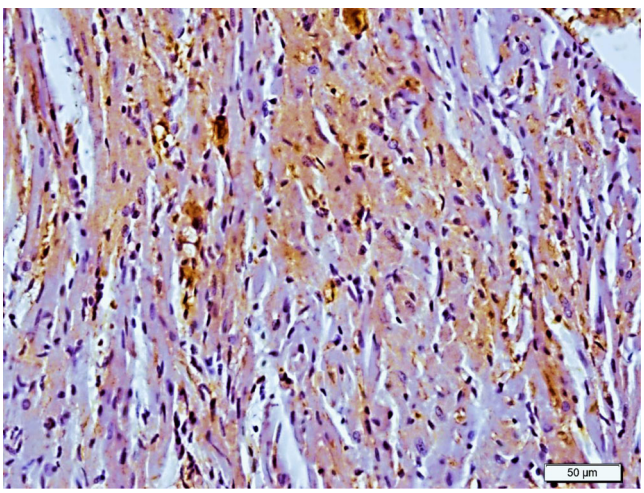



1) Kidney, **IR + β-si**; weak Caspase-3 immunoreactivity.

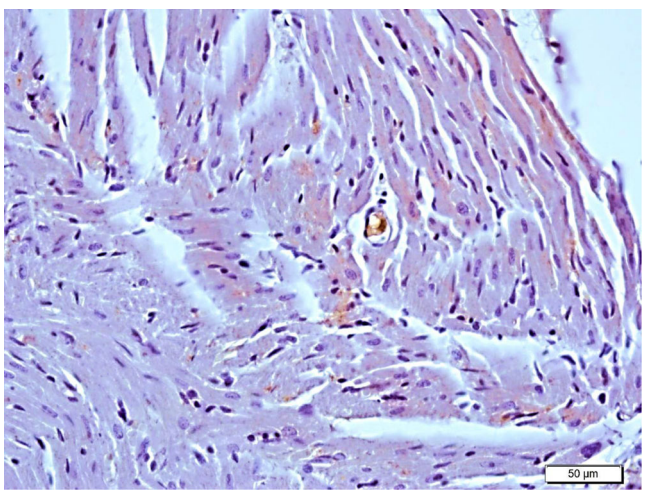
Abbreviation used: IR: Ischaemia/Reperfusion, β-si: β-sitosterol.




2) Heart. **Sham**; Caspase-3 negative,




2) Heart. **IR**; severe Caspase-3 immunoreactivity,




2) Heart. **IR + β-si**; weak Caspase-3 immunoreactivity.

Abbreviation used: IR: Ischaemia/Reperfusion, β-si: β-sitosterol.

**Fig. 3** The effects of β-sitosterol cleaved caspase-3 immunoreactivity on kidney and heart after renal IR injury. 1) Kidney. 2) Heart. Sham, cleaved caspase-3 negative; IR, severe cleaved caspase-3 immunoreactivity; IR + β-si, weak cleaved caspase-3 immunoreactivity. IR ischemia/reperfusion, β-si β-sitosterol

**Table 1** Histopathologic findings and their scores in kidney tissue

| Groups | Sham | IR | IR + β-si |
|---|---|---|---|
| Congestion | _ | +++ | + |
| Hemorrhage | _ | +++ | + |
| Necrosis | _ | +++ | _ |
| Apoptosis | _ | ++ | _ |
| Infiltration | _ | ++ | _ |
| Tubular dilatation | _ | +++ | _ |
| Hyalinization | _ | ++ | _ |

According to histopathologic findings: none (−), mild (+), moderate (++), and severe (+++)

*IR* ischemia/reperfusion, *β-si* β-sitosterol

first time the effects of β-sitosterol on kidney and heart after renal IR protocol in rat. For this, we have employed a female rat model with kidney IR as IR-induced injuries are worsen in male than in female rats (Bazzano et al. 2015; Moslemi et al. 2016). Also, we have pre-applied β-sitosterol to reveal the protective mechanisms of β-sitosterol against renal IR damage (Liu et al. 2020).

The inflammation is implicated after renal IR injury in a mouse model (Xu et al. 2017). However, the mechanisms involved in cardiac remodeling induced by renal IR are not fully understood. In our experimental study, inflammation seems to be a key participant in renal and cardiac pathological changes. This corroborated previous results in the literature, showing that inflammatory cytokines were able to induce renal and cardiovascular inflammation after renal IR (Costa et al. 2019). These new findings lead to the hypothesis of targeting the inflammation as a potential novel therapeutic strategy to alleviate organ damage and dysfunction caused by these conditions. In our study, IR-induced acute kidney damage releases TNF-α and IL-6 that induce a systemic inflammatory response, resulting in inflammatory cells recruitment and remote organ damage. However, β-sitosterol pretreatment reverses the significant increase in TNF-α and IL-6 levels and the reduction in eNOS level triggered by renal IR in

**Table 2** Histopathologic findings and their scores in heart tissue

| Groups | Sham | IR | IR + β-si |
|---|---|---|---|
| Congestion | _ | +++ | + |
| Hemorrhage | _ | ++ | _ |
| Necrosis | _ | ++ | _ |
| Apoptosis | _ | +++ | _ |
| Infiltration | _ | +++ | _ |

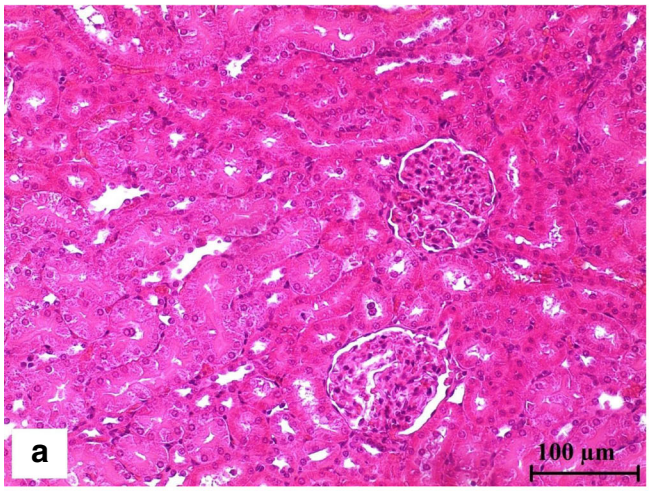
According to histopathologic findings: none (−), mild (+), moderate (++), and severe (+++)

*IR* ischemia/reperfusion, *β-si* β-sitosterol

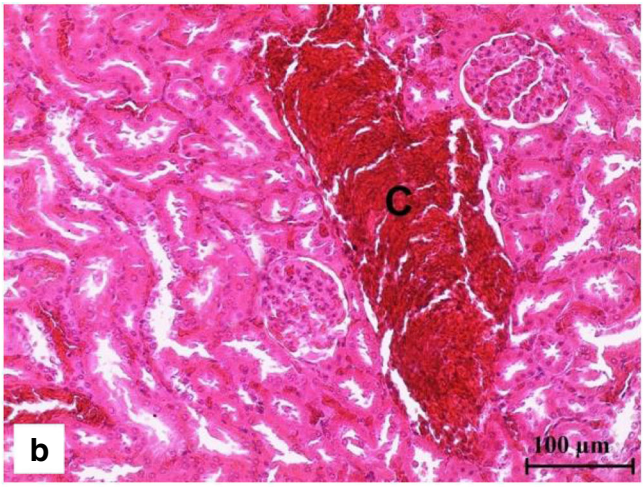
**Fig. 4** H&E staining of rat kidney. Sham: **a** normal structure of kidney. IR, in the IR group, inhomogeneous staining was observed in kidney sections unlike the controls: **b** C congestion. **c** T tubular dilatation; thin arrow, hyalinization; arrow heads, infiltration. **d** C congestion; N necrosis (inside of the squares). **e** C congestion; T tubular dilatation; thick arrow, hemorrhage IR + β-si. **f** Decreased congestion and hemorrhage; C congestion; thick arrow, hemorrhage (scale bars, 100 μm). IR ischemia/reperfusion, β-si β-sitosterol

kidney and heart tissues. In a previous study, administration of β-sitosterol, which degrades neutrophil extracellular traps significantly, prevents diabetes mellitus–induced acute kidney and heart injury (Ikewuchi 2012). Interestingly, β-sitosterol is recently known to alleviate inflammation and can be utilized as a promising future material in wound healing (Mishra et al. 2019). TNF-α levels in renal tissue are markedly lower than the following kidney IR, demonstrating a reduced inflammatory response (Hou et al. 2016). Consistent with these data, we identify that β-sitosterol reduces release of TNF-α, which promotes acute kidney injury–induced acute myocardial injury. Interleukin-6 also mediates myocardial injury following ischemic acute kidney injury and mitigates acute myocardial injury by limiting the subsequent local inflammation (i.e. neutrophils influx and heart damage). Therefore, this cytokine can be an important factor for the physiological and pathological organ damages in animals with IR (Meyer et al. 2017). On the other hand, the formation of NO is implicated in several inflammatory and pathological processes. Especially, this process occurring in renal tissue and heart may cause severe clinical consequences, thereby leading to multiple organ failures (Lei et al. 2018). From a previous study, β-sitosterol could promote insulin sensitivity in rats fed with high-fat diet possibly by increasing eNOS levels (Radika et al. 2013). In the present study, we showed that β-sitosterol was able to improve an inhibition of the eNOS in the heart and kidney tissues of renal ischemic rats. eNOS may play a role in immunomodulatory responses after warm ischemia/reperfusion. In this context, our findings suggested a potential role of β-sitosterol as an in vivo immunomodulator to prevent systemic inflammatory response syndrome after acute kidney damage and the subsequent systemic inflammation-induced acute myocardial injury.

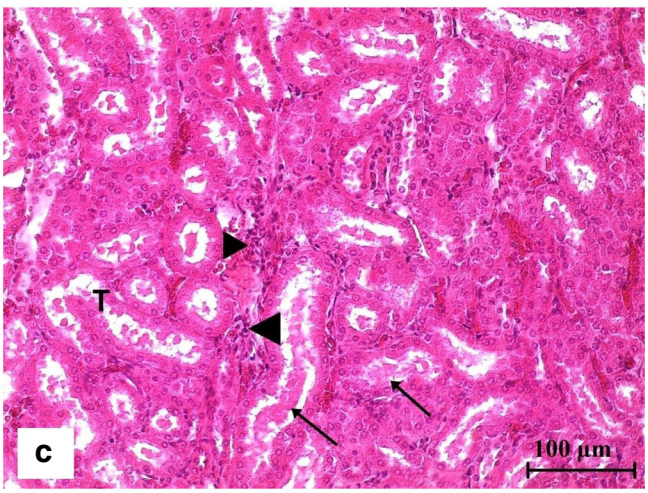
The oxidative stress has been well recognized for being an important and common mechanism that confers the susceptibility to IR-induced cell death (Haushalter et al. 2019). Also, the generation of ROS in renal IR injury is a critical pathological factor contributing to kidney malfunction. On the other hand, increased ROS can be a natural response to cardiac injury including ischemic damage and inhibit cardiomyocyte regeneration. In such a case, the heart is able to regenerate with induction of the antioxidant response (Kloer et al. 2019). Recently, many studies focused in mitigating the organ damages induced by IR injury (Wu et al. 2018). In our study,



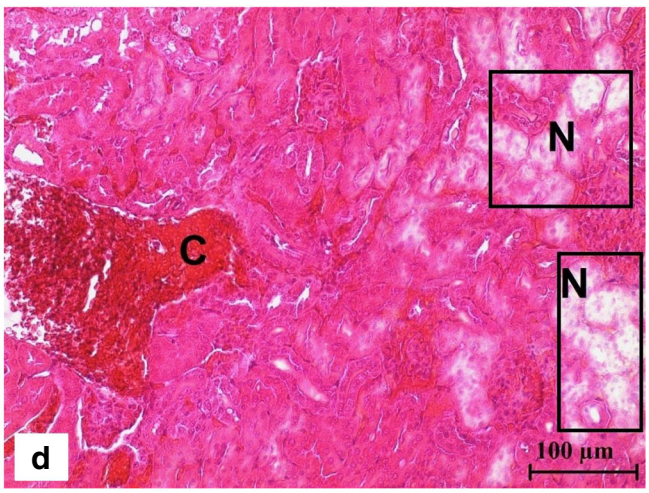

**Sham**; A) normal structure of kidney,



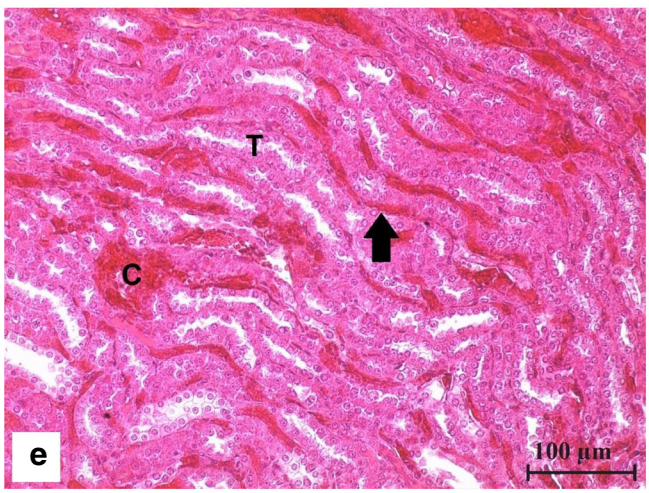

**IR**; In the IR group, inhomogeneous staining was observed in kidney sections unlike the controls B) C: congestion



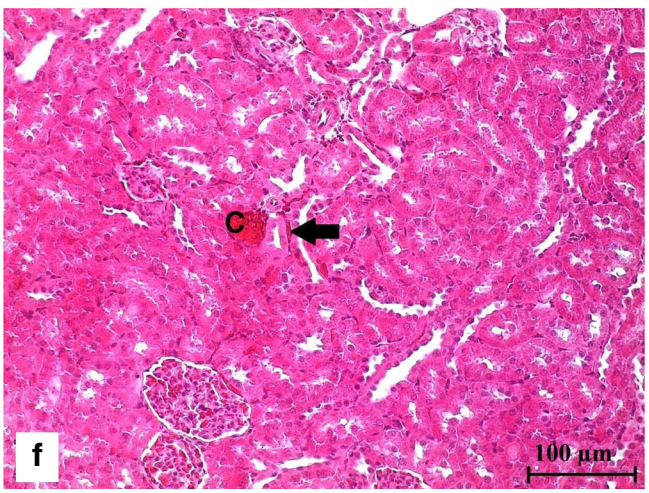

**IR**; C) T: tubular dilatation, Thin arrow: hyalinization, Arrow heads: infiltration,




**IR**; D) C: congestion, N: necrosis (inside of square symbol),




**IR**; E) C: congestion, T: tubular dilatation, Thick arrow: haemorrhage.




**IR + β-si**; F) decreased congestion and haemorrhage, C: congestion, Thick arrow: haemorrhage (scale bars, 100 µm).

Abbreviation used: IR: Ischaemia/Reperfusion, β-si: β-sitosterol.

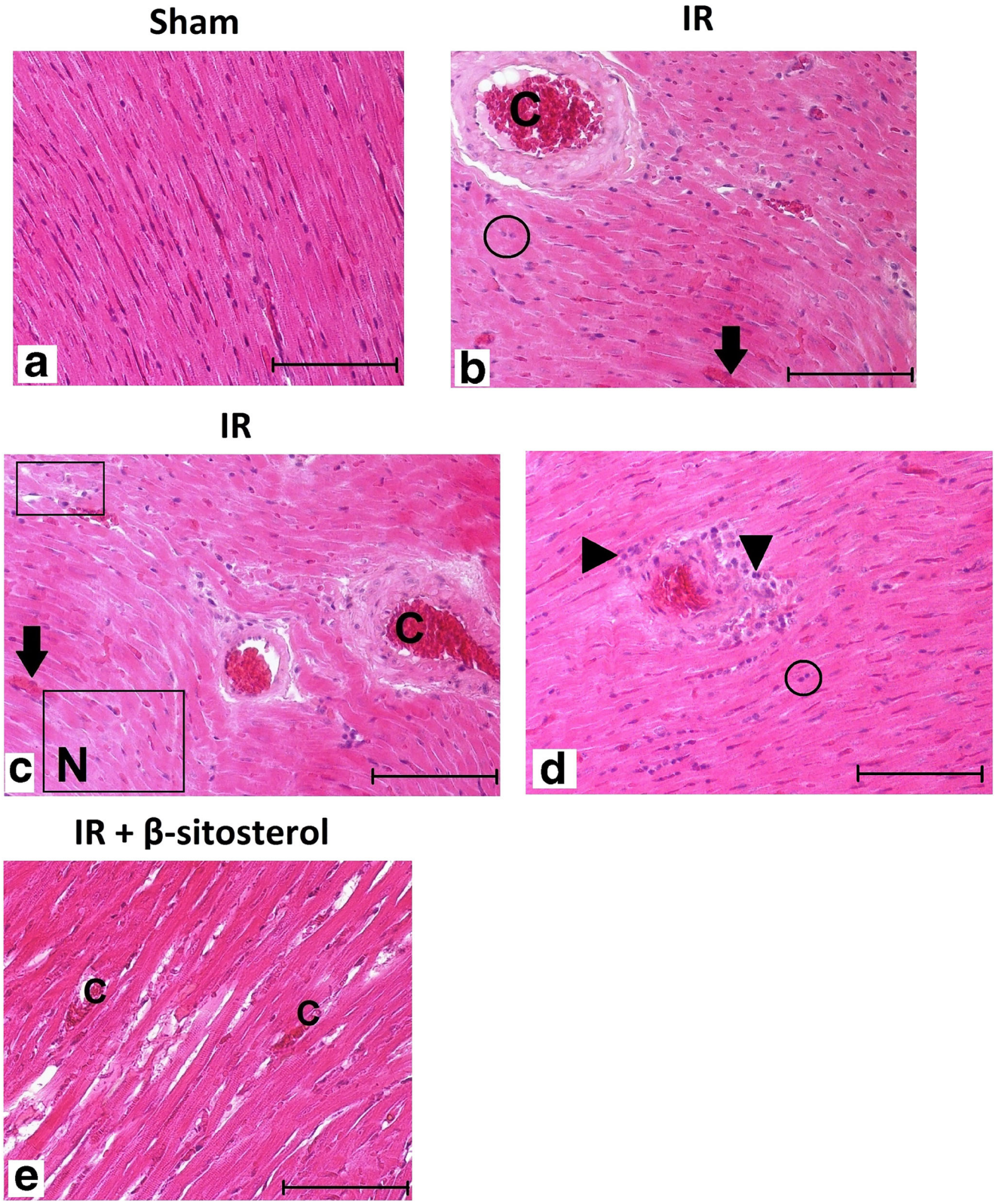



**Fig. 5** H&E staining of rat heart. Sham: **a** normal structure of heart. IR: **b** C congestion; thick arrow: hemorrhage; circle, apoptosis. **c** C congestion; N necrosis (inside of squares); thick arrow, hemorrhage. **d** Arrow heads, infiltration; circle, apoptosis. IR + β-si: **e** Decreased congestion; C congestion (scale bars, 100 μm). IR ischemia/reperfusion, β-si β-sitosterol

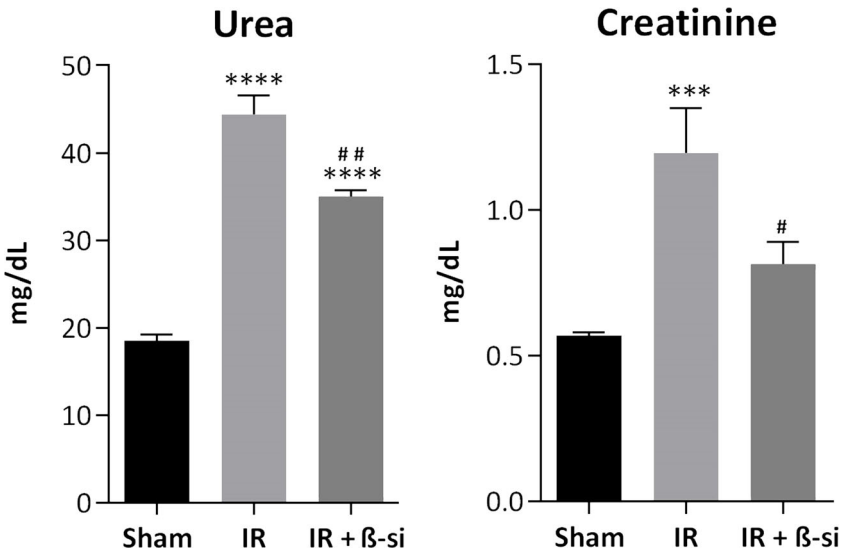



**Fig. 6** The effects of β-sitosterol the serum urea and creatinine after renal IR injury. Data are presented as mean ± SEM ($n = 6$). The asterisks denote significant differences between other studied groups and sham (***$p < 0.0001$, ****$p < 0.0001$). The number signs denote significant differences between other studied groups and renal IR group ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$) by Tukey's multiple range tests. IR ischemia/reperfusion, β-si β-sitosterol

the protective effect of β-sitosterol was manifested in reduced oxidative stress. Thus, this study used the antioxidant parameters as markers for efficacy of β-sitosterol against IR-induced kidney and heart injury. The present study demonstrated that MDA-induced oxidative stress controlled the magnitude of renal IR and the subsequent acute kidney injury–induced acute myocardial damage. Therefore, targeting MDA represented a promising approach to prevent the impact of renal IR injury on distant organs, such as heart (Sherif and Al-Shaalan 2018). The protective effects of β-sitosterol have been reported in chemical-induced oxidative and apoptotic injury for a diversity of tissues and organs in various experimental clinical studies over the years (Parvez et al. 2019). However, any evidence was not available from well-designed experimental studies, which would demonstrate the effects of β-sitosterol in kidney and heart after renal ischemia. Our study could show that β-sitosterol can exert potent protection and reduce myocardial and renal damage following IR via an antioxidant-mediated pathway in the second window of protection. This protection effect was mediated by the activity of SOD enzyme as the first line of defense against oxidative damage. The studies have shown that the activation of antioxidant enzymes increased in remote organs following an initial acute injury to the kidney (Wang et al. 2017). Depletion of glutathione content was also in agreement with previous findings (Zhang et al. 2019). Our study revealed that β-sitosterol prevented IR-induced GSH depletion. Furthermore, β-sitosterol restored the level of MDA to prevent organ damages, which is caused by lipid peroxidation relevant during ischemia/reperfusion injury. GSH is the first nonenzymatic antioxidant that is consumed when the heart and kidney cells are exposed to oxidative stress (Türkoğlu et al. 2017). Increasing evidence has reported that a significant protective effect of GSH was observed by ameliorating MDA in ischemic rats (Erukainure et al. 2019). A study displayed that β-sitosterol suppressed the expression of MDA and supported antioxidant defense in lipopolysaccharide/d-galactosamine-induced acute hepatic injury of antioxidant defense (Yin et al. 2018).

Acute kidney damage causes a significant decrease in renal function. Serum parameters such as urea nitrogen and creatinine are widely measured to assess renal failure in animal models including ischemia/reperfusion injury (Honarpisheh et al. 2018). We found reliable results with these parameters in IR-induced acute kidney injury in rat. A study suggested that IR injury causes renal dysfunction by triggering the evolution of several proinflammatory mediators (Zheng et al. 2019). In another study, IR may lead an increase in the production of free radicals and lipid peroxidation, which can ultimately lead to renal injury and dysfunction (Erpicum et al. 2018). In our study, kidney dysfunction supported these observations as the IR group displayed renal and myocardial changes consistent with this phenomenon-induced oxidative stress and inflammation. Indeed, metabolic acidosis and uremic toxins lead to impaired cardiac mechanical activity and pathological cardiac remodeling, respectively (Lekawanvijit 2018). The results of the present study showed that β-sitosterol improved kidney function after renal IR injury and decreased acute kidney injury. Histological evaluation of the kidney showed structural and tubular aberrations consistent with renal damage after IR rats. The tubular dilatation and inflammation decreased in the IR + β-sitosterol group; however, tubular congestion was slightly observed. According to our study, the increase in oxidative stress and inflammatory response after IR might also cause a bad process, such as apoptosis and necrosis in renal tubules. Findings from our histopathology and immunohistochemical studies also confirmed the protective effects of β-sitosterol on IR-induced

renal and myocardial cell damages and apoptosis. It was reported that β-sitosterol as cholesterol was an important steroid molecule that stabilize phospholipid bilayer of cell membranes (Atolani et al. 2019). On the other hand, the mitochondrial function in IR injury was important and a major intracellular target for cell survival (Kleinbongard and Heusch 2015). β-Sitosterol likely mediated the improved mitochondrial functional ability in target tissues and might be a common regulator of protection from cell deaths (Wong et al. 2014). β-Sitosterol stimulated mitochondrial electron transport and the associated induction of mitochondrial uncoupling (Wong et al. 2016). β-Sitosterol prevented oxidative stress and lipid peroxidation by inhibiting the estrogen receptor antagonists and PI3K inhibitor. β-sitosterol exerted antioxidant activity via estrogen receptor–mediated PI3K/GSK3β signaling (Gutendorf and Westendorf 2001; Shi et al. 2013). β-Sitosterol could improve the promoter activity of Mn SOD but not that of Cu-Zn SOD. β-Sitosterol stimulates antioxidant enzymes by an estrogen receptor/PI3-kinase-dependent pathway, thereby protecting against oxidative stress and lipid peroxidation through modulation of antioxidant enzymes (Vivancos and Moreno 2005).

## Conclusion

The outcome of the current research evidently pointed out the potential of β-sitosterol in renal and myocardial damages. β-Sitosterol prevented IR-induced oxidative stress and pathological changes to kidney and heart. Also, the protective effect of β-sitosterol appeared to act by limiting inflammatory response by preventing the accumulation of TNF-α and IL-6. Thus, the potential of this phytochemical was noteworthy and might serve as a novel target in the treatment of acute organ damages.

**Author contribution statement** All the experiments were carried out by using the infrastructure of the Biology Department of Ataturk University and East Anatolia Research and Application Center (DAYTAM) with the contribution of the authors. Authors declared that the research and the experiments were done by the participation of the researchers in the manuscript.

KK and FG constructed the hypothesis and designed the study. They planned the methodology to reach the conclusion and interpreted the data and finally wrote the paper. OC and AK organized and supervised the histopathological assessments. FA, AY, and KK carried out the oxidative stress and inflammation assays. OC, KK, FG, FA, and AY drafted and revised the work. OC, ZK, FA, AY, and HA performed the experiments and contributed to the data acquisition and data management processes. All authors discussed the results, contributed to the final manuscript, and approved the manuscript. All authors provided critical feedback for the revision of the manuscript. They also reviewed the article before resubmission not only for spelling and grammar but also for its intellectual content.

## Compliance with ethical standards

Experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). All experimental procedures were approved by the Atatürk University Local Ethics Committee for Animal Experiments (No. 194, 04.11.2019).

**Conflict of interest** The authors declare that they have no conflict of interest.

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