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Protective Effect of Luteolin Against Renal Ischemia/Reperfusion Injury via Modulation of Pro-Inflammatory Cytokines, Oxidative Stress and Apoptosis for Possible Benefit in Kidney Transplant

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The acceptances and long-term outcomes of the renal transplantations are seriously jeopardized by inflammatory responses and damage to tissues. The present study intended to explicate the pharmacological effect of luteolin (LT) in renal ischemia/reperfusion (I/R) injury and the possible mechanism of action of LT.

Material/Methods: The effect of LT on the level of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in the homogenates of kidney tissues of male Swiss albino mice was determined after I/R injury. The effect of LT on MDA (malondialdehyde), SOD (superoxide dismutase), CAT (catalase), and glutathione were also identified by enzyme assay. In addition, Western blotting was used to determine the level of Bcl-2, Bax, and caspase-3 in the presence of LT.

Results: The results showed that LT caused significant reduction in the level of TNF-α, IL-1β, and IL-6 compared to the I/R group without LT ($p>0.05$). To further confirm this, the efficacy of LT on the histopathology of I/R injured renal tissues was studied. It was found that LT restored cellular viability of damaged renal tissue. This observation was further confirmed by TUNEL assay, where it was found that LT caused considerable reduction in the population of apoptotic cells. LT pretreatment significantly increased Bcl-2 expression and reduced the level of Bax expression together with a reduction in the level of caspase-3 expression.

Conclusions: Luteolin showed its effect by interfering and attenuating a number of pathways, including pathways for inflammation and apoptosis in renal tissues.

MeSH Keywords: Inflammation • Ischemia • Reperfusion Injury

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Background

The quality of life and survivability of patients affected with serious end-stage renal illness are greatly improved with the renal transplantation [1,2]. Moreover, the survivability of patients is further enhanced by excellent and precise surgical procedures, patient-care, and immunosuppressive drugs [3]. Regardless of these advances, the acceptance and long-term outcomes of the renal transplantation are seriously jeopardized by inflammatory responses and damage to renal tissue [4,5]. This can significantly affected the allograft and the efficiency of the transplanted kidney. Among the causes that affect renal transplants, ischemic/reperfusion (IR) injury is a major cause, resulting from the alteration of blood flow resulting in rapid oxygen deficiency of cells [6,7]. This is initiated with brain death if the donor is dead, or with the closing of the renal artery if the donor is live [8,9]. Other factors are instrumental in damaging the kidney via I/R injury, such as apoptosis and necrosis [10]. Reactive oxygen species (ROS) is considered a major factor in the induction of pathological responses in I/R injury, which leads to inflammatory responses.

Recent studies have confirmed the beneficial role of plant-derived natural products in scavenging the generated free radicals, together with anti-inflammatory actions [11,12]. This pant-based approach has shown promising results in regulating I/R injury and provides the impetus for the search for novel agents from plant origins. Luteolin (LT), a flavonoid obtained from a variety of plants, for instance carrots, peppers, celery, olive oil, peppermint, thyme, rosemary, and oregano, has been extensively studied for its beneficial effect against many ailments because of its strong anti-oxidant and inflammatory properties [13–15]. In the present study, we explored the beneficial effect LT in I/R injury, and its potential to provide beneficial effects for kidney transplants.

Material and Methods

Animals

For this study, 24 healthy male Swiss albino mice (7–9 weeks; 27–30 g) were procured from the Shanghai Experimental Animal Centre of Chinese Academy of Sciences, China. The experiments were performed in agreement with the Institutional Animal Ethical Committee of the Tianjin First Central Hospital, China.

Chemicals and reagents

Luteolin was acquired from Sigma-Aldrich (USA). The antibodies, such as caspase-3, Bcl-2, Bax, and GAPDH were acquired from the Cell Signaling Technology (USA). The ELISA kit was procured from eBioscience (USA).

Animal Study design

Briefly, the male Swiss albino mice were separated indiscriminately into three groups: group 1 was the sham group; group 2 was the ischemia/reperfusion (I/R group); group 3 was the luteolin + ischemia/reperfusion (LT group). The sham group had incision and dissection of the bilateral renal pedicle. Whereas, prior to surgery, the I/R group were administered physiological saline for seven consecutive days before the operative procedure, and the animals in LT group were administered luteolin (100 mg/kg body weight) seven consecutive days before surgery.

Detection of level of cytokines by ELISA

The concentrations of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in the homogenates of the renal tissues from the three groups were determined according to protocol provided by the manufacturers.

Kidney function tests

Briefly, at room temperature the blood samples of the animals of the different groups were centrifuged. The resulting supernatants for each group were pooled, and the concentrations of blood urea nitrogen (BUN) and creatinine in the serum were determined.

MDA, SOD, CAT, and glutathione assay

The effect of LT on MDA (malondialdehyde), SOD (superoxide dismutase), CAT (catalase), and glutathione were identified with the help of enzyme assay kits procured from Jiangsu Chemical Corporation, China, as per the given protocol.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)

Briefly, the staining of paraffin embedded sections of the kidneys were assessed using a TUNEL kit according to protocol as provided by the manufacturer for the *in vivo* determination of apoptotic cells.

Western blotting

The established procedure for Western blotting was used for the estimation of the level of Bcl-2 (B-cell lymphoma 2), Bax (apoptosis regulator), and caspase-3. Briefly, the homogenates of renal tissue were exposed to electrophoresis on SDS-PAGE. The isolated proteins were then moved to nitrocellulose membranes and blocked with defatted milk and incubated with the desired primary antibodies and recorded using the enhanced chemiluminescence system.

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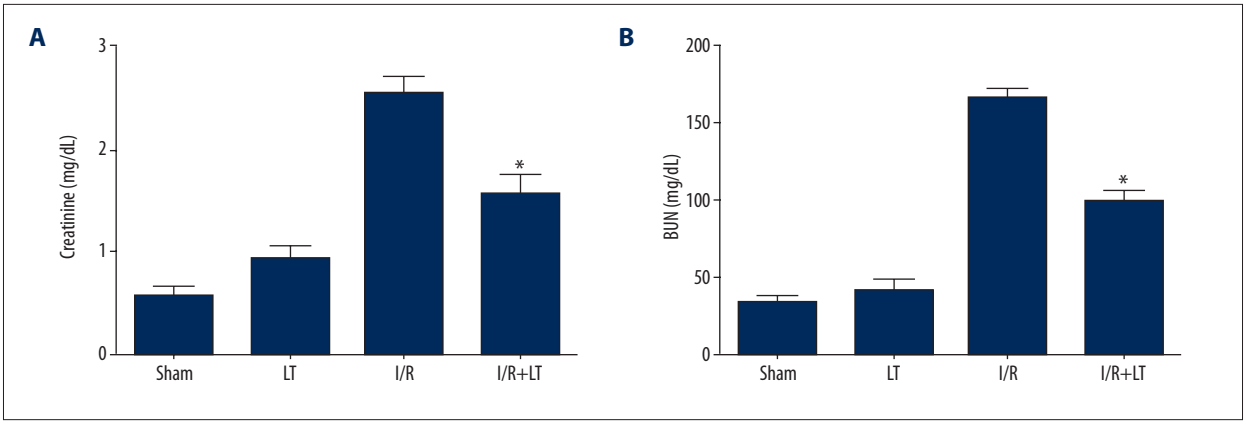

Figure 1. The effect of LT on the serum concentration of (**A**) creatinine and (**B**) BUN after I/R injury; * $p>0.05$.

Statistical analysis

Data are presented as the mean ± standard error of the mean. Statistical analyses were conducted using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The means of the different groups were compared using one-way analysis of variance and Student-Newman-Keuls test. Differences were considered statistically significant when $p<0.05$.

Results

Effect of LT on the serum concentration of creatinine and BUN

As shown in Figure 1, in comparison to the sham group, the serum concentration of BUN and creatinine were enhanced significantly in the I/R group, whereas the level of the serum creatinine and BUN were significantly enhanced in the LT treated group ($p>0.05$). It should be noted that LT treated animals showed considerable effect on the serum level of creatinine and BUN.

Effect of LT on pro-inflammatory cytokines

LT was found to exert considerable influence on the level of various tested pro-inflammatory cytokines. As shown in Figure 2, the animals belong to the IR group exhibited significant increased levels of TNF-α, IL-1β, and IL-6 compared to the sham group. The LT group showed significant improvement in the levels of these cytokines, suggesting its role in improvement of I/R injury might be via an anti-inflammatory effect.

Effect of LT in the oxidant-enzyme system

As shown in Figure 3A, the expression of MDA was found to be elevated in the I/R group compared to the sham group. Moreover, the level of endogenous enzyme system in the I/R group was considerably lower, suggesting a weak antioxidant defense mechanism against free radicals. Treatment with LT caused a decline in the level of MDA, whereas the level of SOD, CAT, and glutathione showed significant improvement compared to the I/R group ($p>0.05$) (Figures 3B–3D).

Histopathological assessment of LT effect

The histopathological examination of renal tissues was performed to validate the protective action of LT on I/R injury. As shown in Figure 4, the histopathological score of the I/R injury group was significantly elevated compared to sham group, showing tubular epithelial cells necrosis (Figure 4C), cellular failure, and increased permeability. Moreover, the LT treated group showed significant improvement as confirmed by mild edema of tubular epithelial cells, minor necrosis, and renal tubule injury (Figure 4D, $p>0.05$).

Evaluation of LT on the Apoptosis

The effect of LT on apoptotic cells were identified, as shown in Figure 5A; the sham group showed few apoptotic cells, whereas, the level of these cells showed significant increase in renal tissues of the I/R group (Figure 5B). It should be noted that the LT group (I/R + LT) showed significantly lower concentrations of apoptotic cells in comparison with the I/R group (Figure 5C).

Assessment of LT on the level of Bcl-2 and Bax

As shown in Figure 6, the levels of Bax and caspase were significantly elevated in the I/R group compared to the sham group. The same pattern of expression was found for Bcl-2 in comparison to the sham group, whereas the LT group showed significant improvement in the level of Bcl-2. The level of the Bax expression was considerably lower when LT was administered.

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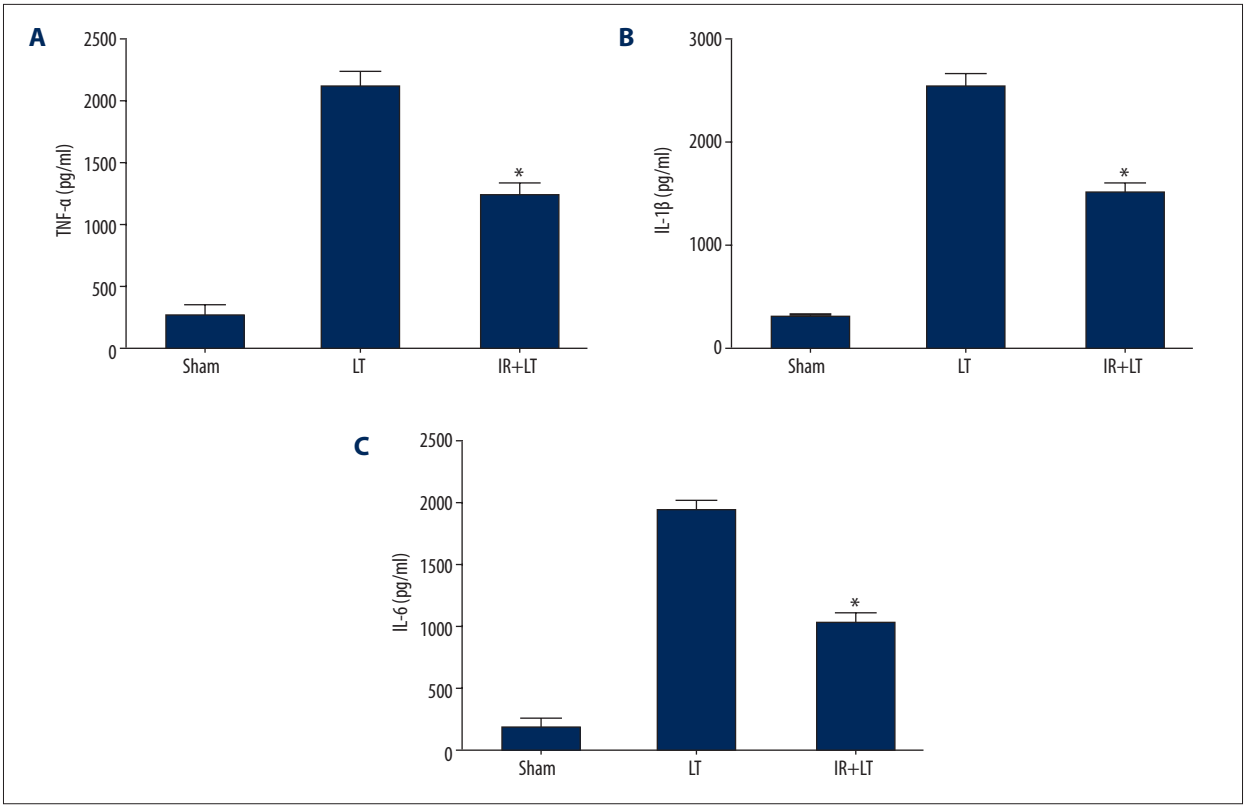

Figure 2. Effect of LT on the levels of (**A**) TNF-α, (**B**) IL-1β, and (**C**) IL-6 in renal tissues after IR injury; * $p>0.05$.

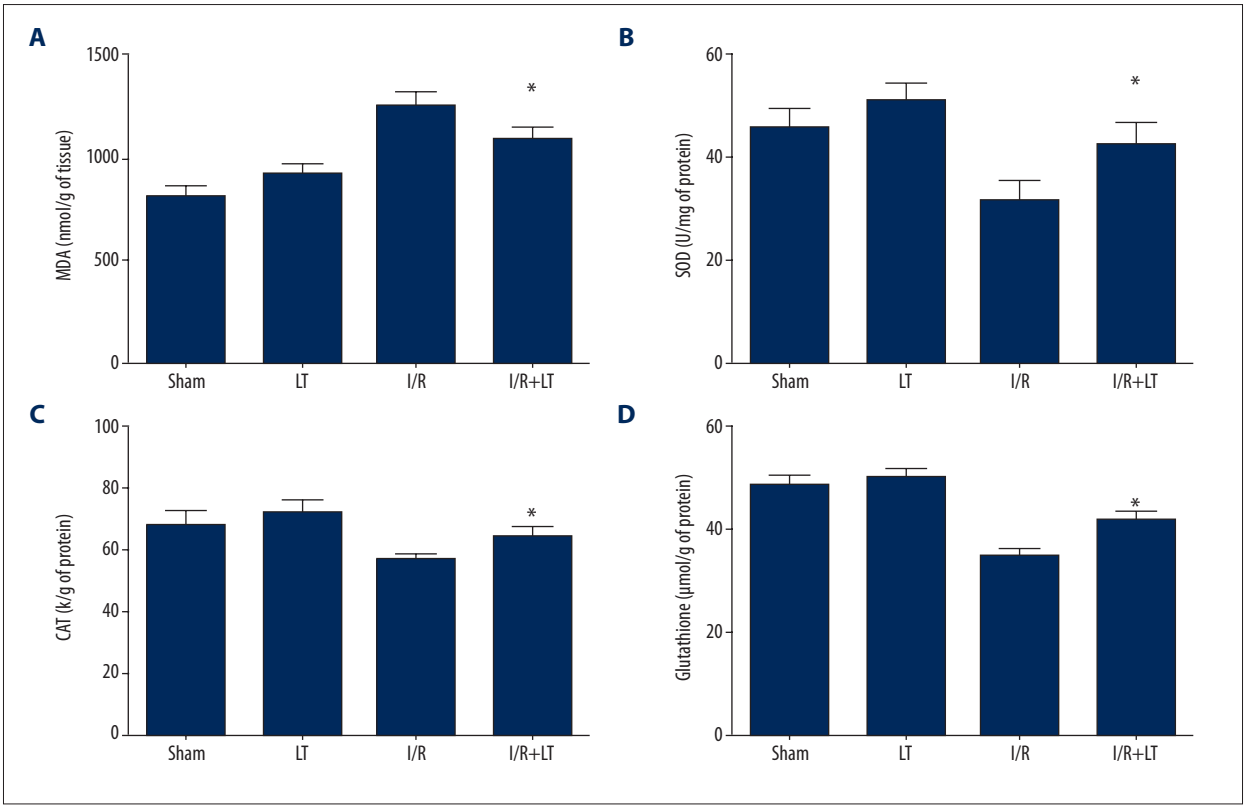

Figure 3. Effect of LT on the level of (**A**) MDA, (**B**) SOD, (**C**) CAT, and (**D**) glutathione in renal tissues after I/R injury; * $p>0.05$.

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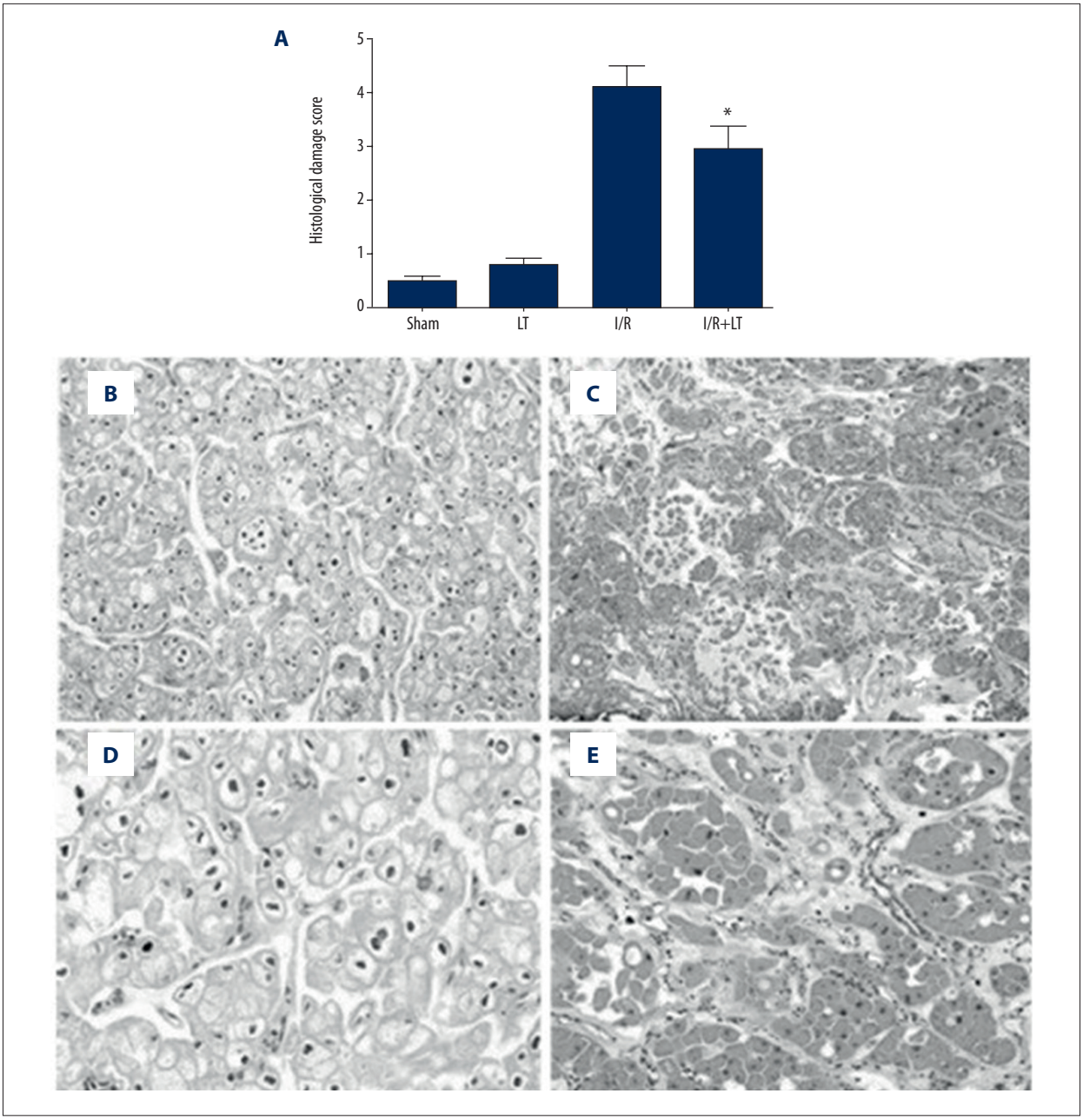

Figure 4. Effect of LT on renal tissue after I/R injury: (**A**) semi-quantitative histopathology scoring, (**B**) sham, (**C**) I/R, (**D**) LT, (E) I/R + LT.

Discussion

A kidney transplant can be seriously affected when the donor kidney has lost its efficacy because of ischemic/reperfusion (I/R) injury [16]. This compromises the allograft function and affects the survivability of the acceptor individual [17]. The I/R injury is considered a frequent cause of acute kidney damage, therefore, any therapeutic modality that can improve this situation can have beneficial effects [18,19]. Plant or plant-based products have provided considerable protective effect in I/R injury because of potent anti-oxidant and anti-inflammatory activities [20,21]. For that reason, the present experiment was conducted to elucidate the effect of luteolin (LT), a plant flavonoid, on I/R injury of the kidney. We selected to work with a mice model of I/R injury because it follows a similar mechanism as that of humans and provides a better understanding of the pharmacological effect of a drug and its mechanism of action [22]. It has been shown that I/R injury causes tissue damage in the renal system initiated via the production of free radicals, increases in calcium ion concentration and infiltration of various pro-inflammatory cytokines inducing apoptosis and necrosis [23]. Therefore, we aimed to determine the effect of LT

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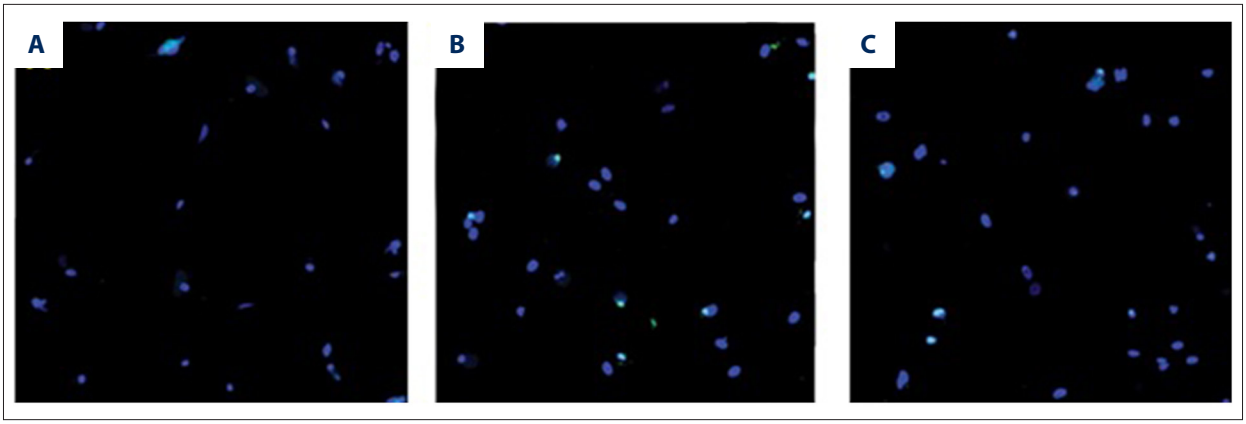

Figure 5. Effect of LT on apoptotic cells as determined by TUNEL assay: (**A**) sham, (**B**) I/R, (**C**) I/R +LT.

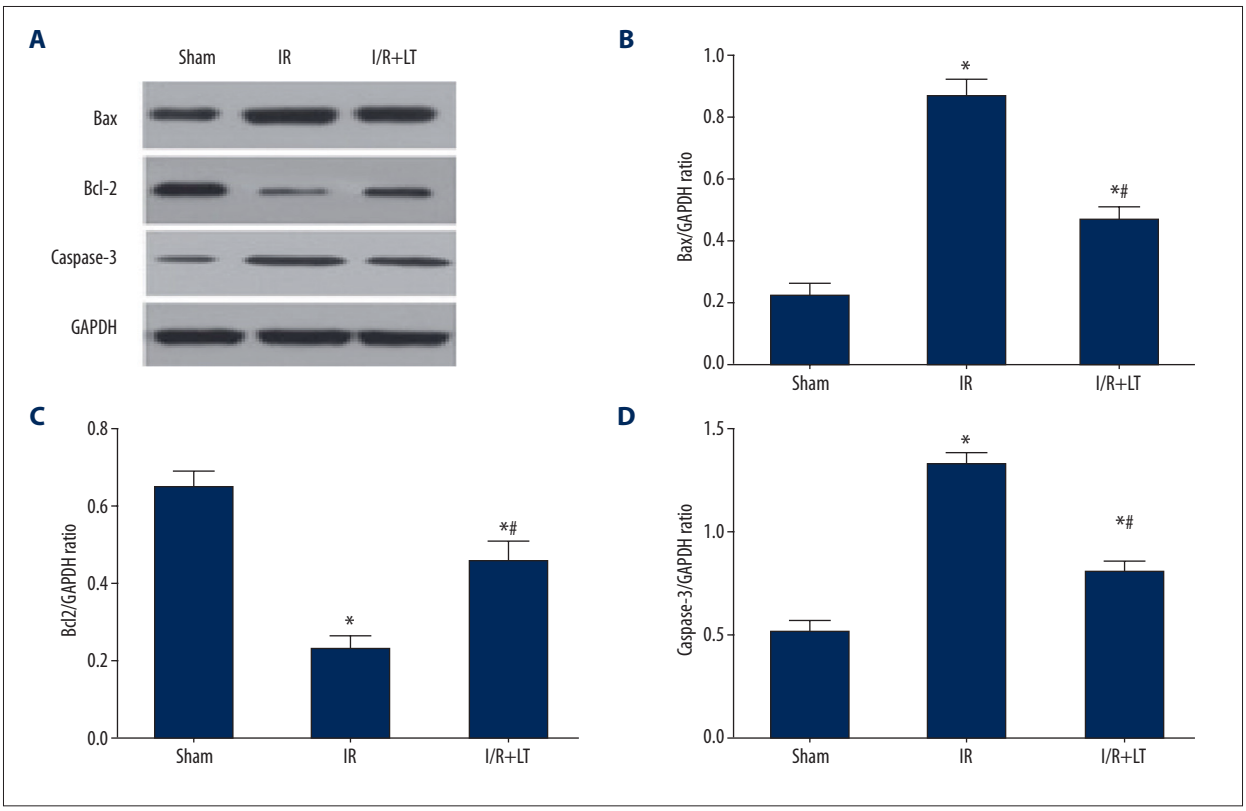

Figure 6. Effect of LT on the protein expression of Bcl-2 and Bax as determined by Western blot analysis.

on these parameters as a mechanistic analysis of its potential beneficial effect against I/R injury. The rise of serum BUN and creatinine indicated that the renal system had some complications affecting the normal function of the kidney related to internal damage. Various studies have confirmed that in the case of I/R injury, the level of these biomarkers is significantly elevated in I/R injured animals compared to controls [24]. Thus, the effect of LT on these biomarkers have been studied, and studies have found that LT causes significant reduction of serum BUN and creatinine, which further suggests a beneficial role in renal tissues with I/R injury. Earlier studies have confirmed the role of LT on the level of various pro-inflammatory cytokines together with anti-oxidant activity in a variety of pathological conditions [25,26]. LT has been shown to have a protective effect in cardiac reperfusion injury via inhibition of ROS-activated MAPK pathway and have a potent antioxidant effect. Similarly, in the present study, LT was shown to enhance free radical scavenging ability via modulation of MDA, SOD, glutathione, and CAT indicating a protective mechanism against I/R injury. The level of various pro-inflammatory cytokines released from activated endothelium and mast cells have been found significantly elevated in I/R injury, suggesting

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its role in promotion of inflammatory conditions [27]. These released cytokines mediate their inflammatory response via NF-κB and exacerbate the tissue injury. Therefore, the effects of LT on the levels of TNF-α, IL-1β, and IL-6 were investigated in our study. Our results showed that LT caused significant reduction in the level of TNF-α, IL-1β, and IL-6 compared to the I/R group ($p>0.05$). To further confirm the efficacy of LT, the histopathology of I/R injured renal tissues were also studied. It was found that LT restored cellular viability of the damaged renal tissues, which was confirmed by the restoring of the original architecture of the cells via reduction of mild edema of tubular epithelial cells, minor necrosis, and renal tubule injury. This observation was further confirmed by TUNEL assay, where it was found that LT caused a considerable decrease in the population of apoptotic cells. Various studies have shown an interrelated relationship between apoptosis and the level of Bcl-2 and Bax [28]. It has been shown that Bcl-2 can impede the production of free radicals, emission of intracellular Ca^{2+}, and porousness of mitochondrial membrane [29,30]. Bax has been shown to promote apoptosis [31]. Consequently, the relationship of Bax to Bcl-2 regulates how cells endure or experience apoptosis [32,33]. Thus, we studied the effect of LT on the expression of these proteins and found that LT pretreatment significantly increased the level of Bcl-2 and reduced the level of Bax. This was further potentiated by the fact that LT also caused a reduction in the level of caspase-3, which ultimately reduced the apoptosis of the renal tissues.

Conclusions

As a concluding remark, we wish to report the protective and beneficial effect of luteolin on renal ischemic/reperfusion injury, which is a grave concern for kidney transplantation efficiency. Luteolin exerts its effect by interfering and attenuating a number of pathways, including inflammation and apoptosis pathways in renal tissues.

Conflict of interest

The authors have declared no conflict of interest.

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