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Luteolin alleviates renal ischemia-reperfusion injury in streptozotocin induced diabetic rats by inhibiting metalloenzymes expression

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ABSTRACT

Diabetes patients are more prone to acute kidney injury (AKI). Endopeptidases known as matrix metalloproteinases (MMPs) cause extracellular matrix destruction and are responsible for ischemic organ damage. Diabetic nephropathy (DN) affects almost one third of all diabetic patients. MMP-2 and MMP-9 lead to the breakdown of the basement membrane of the glomeruli and thereby the advancement of ischemic injury in diabetes. In addition, histone deacetylase-2 (HDAC-2) is the primary regulator of important signalling processes in the diabetic kidney. A possible treatment approach for diabetic kidney preservation is the flavonoid luteolin (LT), which has anti-inflammatory and antioxidant effects. Our aim was to investigate the renoprotective potential of LT in diabetes by modulating MMP-2, MMP-9 and HDAC-2 activity. The expression of MMP-2, MMP-9 and HDAC-2 were statistically higher in streptozotocin-induced diabetic rat renal homogenate after renal ischemic reperfusion injury. These changes were reversed with 2 weeks of pre-treatment with LT (50 mg/kg po). In diabetic rats, pre-treatment with LT significantly reduced oxidative stress, inflammation and fibrosis compared to control animals. Preventive LT prior to renal ischemia showed improvement in body weight, kidney weight/body weight ratio, reversal of renal injury and biochemical changes with lower activity of malondialdehyde (MDA), myeloperoxidase (MPO), hydroxyproline (HP), pathological damage and fibrosis in renal tissue. Our data imply that LT prevents DN in rats by inhibiting MMP-2, MMP-9 and HDAC-2 expression, as well as by lowering the indices of oxidative stress, pro-inflammatory factors and fibrosis.

INTRODUCTION

Acute kidney injury (AKI) is a ubiquitous condition that has a high mortality rate. Various medical conditions, such as shock, sepsis, vascular occlusion, and renal transplantation, can result in AKI owing to renal ischemia and reperfusion (I/R) [1]. A variety of intricately interwoven pathophysiological mechanisms impact the pathophysiology of I/R damage, including excess calcium, oxidative stress, inflammation, endoplasmic reticulum stress, mitochondrial dysfunction and apoptosis [2]. Patients with diabetes have a higher risk of renal I/R damage, which can have substantial consequences for morbidity and death in clinical settings [3]. Ischemia induces a cellular buildup of xanthine oxidase and hypoxanthine due to a lack of oxygen. The most deleterious effect of reperfusion is caused by the formation of very harmful free radicals, which are produced in enormous amounts once oxygenation is restored. Damage is exacerbated by neutrophil infiltration, cytokine activation, cellular apoptosis, necrosis and microvascular damage [4]. Renal ischemia-reperfusion injury increases the permeability of the endothelial barrier due to degradation of the perivascular matrix [5].

Diabetes exacerbates oxidative stress, inflammation and apoptosis, which worsen renal ischemia and reperfusion damage in rats. Pre-treatment with antioxidants may lessen renal I/R damage [6]. Zinc-dependent endopeptidases, called matrix metalloproteinases (MMPs), degrade and alter the extracellular matrix (ECM) and are implicated in ischemic organ damage [7]. The ECM components of the basement membranes, such as type IV collagen, laminin and fibronectin, are degraded by MMP-2 (72 kD gelatinase A) and MMP-9 (92 kD gelatinase B). MMP substrates can also disrupt both signaling molecules and cell adhesion

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molecules. MMP activity can be reduced via alternative methods using tissue metalloproteinase inhibitors (TIMP) [8]. MMP-2 and MMP-9 have been implicated in the pathogenesis of ischemia-reperfusion damage in various organs, including the kidney [9-11]. Intracellular MMP-2 activity within cardiomyocytes cleaves cardiac troponin I because of ischemia-reperfusion injury [12,13]. After ischemia-reperfusion, MMP-2 and MMP-9 are increased in the kidney, and MMP activation modifies renal microvascular permeability [7,14,15].

An experiment conducted by Kunugi *et al*., showed that when the duration of ischemia increased between 30 and 120 minutes, the activity of MMP-2 and MMP-9 was significantly high after 6 hours, and the severity of AKI increased after 24 hours. MMP inhibitors reduced acute tubular injury (ATI) and improved renal dysfunction at 24 hours [7]. Moreover, increased renal sensitivity to ischemia/reperfusion (I/R) injury results in a progressive injury with end-stage renal failure in streptozotocin-diabetic rats [16]. Matrix metalloproteinase-2 is essential in the pathophysiology of kidney disease, where increased MMP-2 expression is seen in several experimental models of renal injury, including ischemia/reperfusion injury in the context of the kidney [7,17,18]. Ischemia followed by reperfusion causes proteolysis and degradation of the extracellular matrix (ECM) in renal tubular epithelial cells, according to published data [19]. MMP-2 and MMP-9 are involved in cleaving and altering the ECM. Acute MMP-9 build-ups produced by neutrophils increases inflammation and exacerbates graft damage [19]. These reported results provide a strong basis for understanding the function of MMPs in diabetic kidneys and how an increase in the ischemia situation leads to diabetes progressing to diabetic nephropathy.

HDAC can effectively regulate the transcriptional network. Even though there have been few publications on the role of HDACs in controlling crucial signalling mechanisms in the diabetic kidney, this fact has recently received more attention. This idea has been supported by both *in vitro* and *in vivo* studies. Noh and co-workers initially looked at the function of HDAC in diabetic nephropathy in 2009, where the activity of the HDAC-2 isoform increased in the kidneys of streptozotocin-induced diabetic rats, type 2 diabetic *db/db* mice, and proximal tubule lineage NRK-52E cells exposed to the profibrotic cytokine transforming growth factor beta1 (TGF-β1), confirming the role of Histone deacetylase-2 in diabetes- and TGF-β1-induced renal injury [20]. It is demonstrated that inhibiting HDAC reduces ischemia damage in the brain, heart, retina and AKI [21-23]. Reduced ischemia-induced retinal damage can be achieved by inhibiting HDAC-2 expression [22]. HDAC inhibition also accelerates recovery after AKI [24]. The CoREST complex is stabilized in renal tubular cells by HDAC-2 targeting, which also protects against renal ischemia/reperfusion damage and suggests endothelin as a possible downstream mediator of renal injury [23]. The suppression of HDAC-2 by sodium butyrate in *db/db* animals and HG-induced NRK-52E cells, which are frequently employed as diabetes models, lowers kidney cell apoptosis through an antioxidant mechanism [25]. In a recent study, it was shown that short-chain fatty acids (SCFAs) can reduce renal fibrosis and increase autophagy of renal tubular cells in both diabetic C57BL/6 mice and Akita mice by inhibiting the HDAC-2/ULK1 axis [26]. The previously reported research reaffirms the function of HDAC in diabetic kidney disease and ischemia damage, which lead to severe diabetes-related conditions.

Flavonoids can be used to treat DN by reducing oxidative stress and inflammatory responses due to their protective properties against DN [27-29]. More studies are, however, needed because of the scanty information available in this area on the complications associated with diabetes and AKI. Luteolin is a flavonoid that has anti-inflammatory, antioxidant, anti-apoptotic, anti-allergic and anti-uric acid effects [30,31]. Some chronic inflammatory diseases, including diabetes-related diseases such as colistin/cisplatin-induced nephrotoxicity, diabetic nephropathy and lipopolysaccharide (LPS)-induced acute kidney injury, have been treated with LT, which shows a protective role according to previous research [32-37].

Even after careful glucose control and consuming antihypertensive drugs, diabetic kidney damage can develop and worsen [38]. Glucagon-like peptide-1 (GLP-1) receptor agonists, sodium-glucose transporter 2 (SGLT-2) inhibitors, renin-angiotensin system (RAS) blockers and other drugs are now used to treat DN [39]. Despite the availability of these treatment alternatives, DN cannot be fully treated with today's drugs, suggesting the need for a deeper understanding of the molecular processes underlying DN pathogenesis. The current study aimed to determine the effect of LT pre-treatment on the expression of MMP-2, MMP-9 and HDAC-2 proteins on renal ischemia damage in streptozotocin-induced diabetic rats, as well as to investigate the effect on biochemical, oxidative stress, pro-inflammatory mediators and pathophysiological changes associated with LT pre-treatment in DN rats.

MATERIAL AND METHODS

Chemicals

Streptozotocin was obtained from Sigma Chemicals Ltd. in Germany, while LT was purchase from Santacruz Biotechnology Ltd. in Dallas, Texas. All other chemicals were procured from SRL in Mumbai, India. Erba Diagnostic supplies blood biochemical parameter monitoring kits. MMP-2 (KLR0315), MMP-9 (KLR0321) and HDAC-2 (KLR1410) ELISA kits were bought from Krishgen Biosystem in Mumbai, India.

Animals

Male Wistar rats weighing 200-250 grams were purchased from the National Institute of Biosciences, Pune, and acclimated for two weeks under standard laboratory settings before the start of the studies. Throughout the experiment, the animals were kept in a room with proper ventilation (16-18 air changes/hour), controlled temperature (20-24^{o}C), relative humidity (45-65%) and a light/ dark cycle of 12 hours. The animals had free access to feed pellets and clean water. All experimental procedures were conducted as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA),

India. The Institutional Animal Ethics Committee of Dr. M.S. Gosavi College of Pharmaceutical Education and Research, Nashik approved all experimental procedures for animal use and care (Ref. No. MSGCOPER/IAEC/01/2016).

Experimental Protocol

Male Wistar rats were injected with streptozotocin (55 mg/kg i.p.) formulated in 0.1 M ice cold citrate buffer pH 4.5, to cause diabetes. Blood glucose levels were measured one week after injection using Accurex glucose testing kits. Animals with blood glucose levels greater than 300 mg/dl were selected and divided into four groups consisting of six animals each and treatment were started after confirmation of hyperglycaemia. For the control groups, sodium carboxymethyl cellulose (Na-CMC) in water (0.5% w/v) was used as the vehicle, while luteolin suspended in Na-CMC in water was used for the LT treatment group. Preliminary studies conducted in our laboratory revealed promising results with 50 mg/kg of luteolin from the three doses selected (25, 50 and 75 mg/kg p.o.). Therefore, a dose of 50mg/kg of luteolin was chosen for future studies. The following groups were given pre-treatment for 14 days and 1 hour before ischemia.
Group I: diabetic IR control group (diabetic rats received Na-CMC in water + I/R);
Group II: sham group (diabetic rats received Na-CMC in water);
Group III: luteolin group (diabetic rats received LT (50 mg/kg p.o.) in Na-CMC in water + I/R).

On the day of surgery, animals in groups I and III were anaesthetized with sodium pentobarbitone (50 mg/kg i.p.), the left renal artery was isolated and clamped with a rat bulldog clamp for 30 minutes. After the clamp was removed, the incision was sutured, and the stitches were treated with povidone-iodine ointment. The procedure was the same in Group II except that the renal artery was not clamped. After 24 hours of reperfusion, the animals were placed in the cage to recover before being euthanized. The body weight of the rats was checked before and after the experiment. Prior to euthanisation, blood was collected from the retroorbital plexus, and the serum was separated by centrifuging the blood at 7000 rpm for 15 minutes at 4°C. The serum collected was analysed for glucose (BSL), creatinine (Cr), blood urea nitrogen (BUN), uric acid, total protein (TP), serum albumin, total cholesterol (TC) and alkaline phosphatase (ALP). Urine was also collected for urine volume and creatinine clearance. The rats were euthanized by CO2 euthanasia, and the left kidneys were removed and weighed to calculate the kidney weight/animal weight ratio and then stored for biochemical estimation (as set out in the following sections). For histological analysis, a portion of the kidney was preserved separately in 10% buffered formalin and hematoxylin and eosin (H&E), and Masson trichome (MT) staining was done [7,16,40].

Measurement of biochemical parameters

Erba Diagnostic Kits were employed to measure fasting blood glucose (FBG), serum creatinine, blood urea nitrogen (BUN), uric acid, serum albumin, total cholesterol (TC), total protein (TP) and alkaline phosphatase (ALP) [41].

Renal tissue homogenate preparation

After the rat sacrifice, kidneys were extracted and washed using PBS to remove excess blood. The kidneys were placed on an ice-cold Petri dish, and thin slices of tissue were extracted from the specimens employing a surgical scalpel and promptly dabbed on filter paper. The kidneys were minced using a glass homogenizer and combined with a 10% phosphate-buffer solution at pH 7.4. The tissue was homogenised using a Remi refrigerated centrifuge with a Teflon pestle, with 25 strokes applied at 7000 g for 20 minutes at 4°C. A transparent liquid was used to quantify metallozymes and other parameters using commercially available kits and following the instructions provided by the manufacturer.

Estimation of malondialdehyde (MDA), Myeloperoxidase (MPO) and Hydroxyproline (HP) content in the kidney tissue

Using the thiobarbituric acid reaction method of Okhawa *et al*. [42], the level of MDA was used to measure lipid peroxidation. MPO activity was measured using method of Suzuki *et al*. [43]. The hydroxyproline content was determined using the Woessners technique [44]. These methods were standardized in our laboratory, as described previously [45].

Estimation of MMP-2, MMP-9 and HDAC-2 expression

According to the manufacturer's instructions, MMP-2, MMP-9, and HDAC-2 expression were estimated in kidney homogenate and cell culture supernatant using a commercially available enzyme-linked immunosorbent assay (Rat MMP2/gelatinase A; Rat MMP-9/gelatinase B and Rat HDAC-2 of GENLISA™ ELISA kits).

Histopathological study

The kidneys were removed and stored in 10% buffered formalin before being divided in half parallel to the major axis. They were then washed and immersed in isopropyl alcohol, xylene, and paraffin for 24 hours for microscopic examination. For histological evaluation, 5 µm thick paraffin-embedded tissue sections were cut and stained with hematoxylin and eosin (H&E) after deparaffination and Masson's trichome (MT) staining to examine gross morphology and identify fibrosis. The distribution of collagen was measured using Masson's trichome staining method. Each specimen was evaluated for histopathologic abnormalities in the glomeruli, tubules, interstitium and blood arteries. The changes in the kidney were evaluated using a scale to determine the severity of neutrophil infiltration, vascular alterations and tubular necrosis. The scale included none (-), mild (+), moderate (++), and severe (+++) for necrotic lesions affecting 60% or more of the affected area [46-48].

Statistical analysis

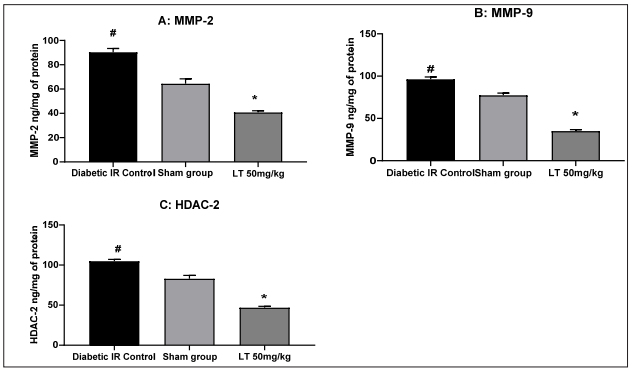
All values were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism (Graph Pad Software Inc., version 6.0 for Windows, San Diego, California, USA). One-way analysis

of variance (ANOVA) followed by Dunnett's test was used for data analysis; $p < 0.01$ was considered statistically significant.

RESULTS

Effect of luteolin on MMP-2, MMP-9, and HDAC-2 expression in renal homogenate in renal ischemia injury in STZ-induced diabetic rats

The diabetic rats exposed to renal ischemia injury exhibit changes in the expression of metallozymes in the kidney homogenate. The expression of MMP-2, MMP-9, and HDAC-2 was found to be significantly higher in the diabetic control group compared to the diabetic sham group ($p < 0.01$). The expression of MMP-2, MMP-9, and HDAC-2 in the kidney tissue of diabetic rats was significantly reduced ($p < 0.01$) following LT pre-treatment for two weeks before renal ischemia, as compared to the diabetic control group (Figure 1).



Values are expressed as mean ± S.E.M., using one way ANOVA followed by Dunnett's test, #p<0.01 when diabetic control group is compared with diabetic sham group, *p<0.01 when LT treated group is compared with diabetic control group (n=6)

Figure 1. Effect of luteolin on A: MMP-2, B: MMP-9 and C: HDAC-2 expression renal homogenate of diabetic rats exposed to ischemia reperfusion injury

Effect of luteolin on biochemical parameters in renal ischemia injury in STZ-induced diabetic rats

The results of the different parameters of renal function following pretreatment with LT at a dosage of 50 mg/kg in diabetic rats with renal ischemic injury are presented in Table 1. The diabetic control group exhibited significantly elevated levels of serum creatinine, BUN and uric acid, suggesting a severe impairment in renal function. The pretreatment with LT for a duration of two weeks with I/R resulted in a significant improvement in various parameters in diabetic rats. These parameters include body weight, serum creatinine, BUN, TC, TP, uric acid, ALP and serum albumin levels. The administration of LT brought these levels closer to the normal range. In contrast to the control group, the LT pretreatment exhibited a marginal but significant reduction in BSL ($p < 0.01$).

In our study, rats administered STZ had a mortality rate of 6.67% within one week, while the diabetes control group had a rate of 6.67% two weeks after injection. However, in the diabetic sham rats that received the vehicle orally, the mortality rate was significantly lower at 3.33%. The overall mortality rate was 16.68%.

Effect of luteolin on body weight, kidney weight/body weight and blood glucose

The mean body weight of the diabetic rats exposed to renal ischemia exhibited a significant decrease as compared to the diabetic sham group ($p < 0.01$). The body weight significantly increased in the LT-pretreated groups, indicating a positive influence on body mass. The kidney weight/body weight ratio was observed to be significantly higher in the diabetes control group when compared to the sham group. In contrast, the pretreatment with LT group displayed significantly lower levels of kidney weight/body weight than the diabetic control group ($p < 0.01$). Blood glucose levels also demonstrated a significant increase in diabetic rats after two weeks of STZ therapy followed by I/R as compared to the diabetic sham group ($p < 0.01$). However, LT pretreatment prior to renal ischemia marginally but significantly decreased hyperglycemia in the diabetic rats (Table 1).

Effect of luteolin on renal function

Serum levels of creatinine (Cr), blood urea nitrogen (BUN) and uric acid were all considerably greater in the diabetic rats that underwent renal ischemia as compared to the sham group ($p < 0.01$), suggesting a decline in renal function (Table 1). In contrast, creatinine clearance was significantly lower in the diabetic control group than in the sham group (Table 2). When compared to the diabetic control group, the aforesaid renal indices were significantly reduced ($p < 0.01$) after pretreatment with LT at a dosage of 50 mg/kg for 2 weeks prior to ischemia.

Effect of luteolin on TP, Serum Albumin, TC and ALP

The diabetic rats that underwent renal ischemia exhibit a significant reduction in total protein and albumin in the blood as compared to the diabetic sham group due to renal impairment. In contrast, the levels of total protein and albumin in the blood were significantly increased in rats who received LT pre-treated for two weeks before renal ischemia as compared to the diabetic control group ($p < 0.01$). The statistical analysis revealed a significant disparity in the levels

Table 1. Effect of Luteolin on renal function, blood sugar level and lipid profile in STZ induced diabetic rats

Groups	Body weight in gram	Kidney weight/Body weight	BSL mg/dl	Serum Creatinine mg/dl	BUN mg/dl	Uric acid mg/dl	TC mg/dl	TP g/dl	ALP (U/L)	Serum Albumin g/dl
Diabetic IR Control+ Vehicle	182.7±2.57#	11.27±0.26#	387±6.12#	1.59±0.03#	43.85±1.65#	2.81±0.11#	93.8±2.7#	3.56±0.12#	186±3.7#	3.3±0.1#
Diabetic Sham + No I/R + Vehicle	197.7±1.91	7.89±0.33	345±3.5	0.99±0.03	31.95±0.95	2.04±0.05	69.2±1.5	5.24±0.13	166±2.5	4.2±0.2
Diabetic +I/R LT 50 mg/kg p.o	220.3±2.97*	6.34±0.23*	357±5.4*	0.81±0.02*	27.25±1.14*	1.15±0.03*	53.2±1.8*	5.64±0.1*	119±4.0*	4.8±0.1*

Note:
Values are expressed as mean ± S.E.M. (n=6), using one-way ANOVA followed by Dunnett's test
#p<0.01 when the diabetic IR control group is compared with the diabetic sham group
*p<0.01 when the LT treated group is compared with the diabetic IR control group (n=6)

of TC and ALP between the diabetic control group and the diabetic sham group (p <0.01). This finding implies that diabetic rats encountered challenges related to lipid metabolism. The administration of LT led to a significant reduction in TC and ALP levels in comparison to the diabetes control group (p <0.01) (Table 1).

Effect of luteolin on urine parameter

The urine volume (in ml) of diabetic rats after 24 hours of renal ischemia was found to be significantly higher (<0.01) as compared to that of the diabetic sham group. When diabetic rats were pretreated with LT (50 mg/kg) before ischemia, a significant decrease in urine volume was observed (p <0.01) compared to the diabetic control group (Table 2).

Table 2. Effect of luteolin on urine parameters

Groups and Treatment	Urine Volume ml	Creatinine Clearance ml/min
Diabetic IR Control + Vehicle	45±1.52#	0.662±0.04#
Diabetic Sham + No I/R + Vehicle	35±1.04	0.848±0.02
Diabetic +I/R LT 50 mg/kg p.o	22±1.80*	1.057±0.01*

Note: Values are expressed as mean ± S.E.M. (n=6), using one-way ANOVA followed by Dunnett's test. #p<0.01 when the diabetic IR control group is compared with the diabetic sham group, *p<0.01 when the LT treated group is compared with the diabetic IR control group (n=6).

Effect of luteolin on MDA, MPO and Hydroxyproline content in renal homogenate

Malondialdehyde (MDA), a diagnostic marker of oxidative stress; myeloperoxidase (MPO), a marker of inflammation; and hydroxyproline (HP), a signal of fibrosis, were all significantly (p <0.01) higher in the diabetic rats exposed to renal ischemia as compared to the diabetic sham group. Pretreatment with LT (50 mg/kg) two weeks prior to renal ischemia resulted in a significant reduction (p <0.01) in the content of MDA, MPO, and in the expression of hydroxyproline in the renal homogenate, as compared to the diabetic control group (Table 3).

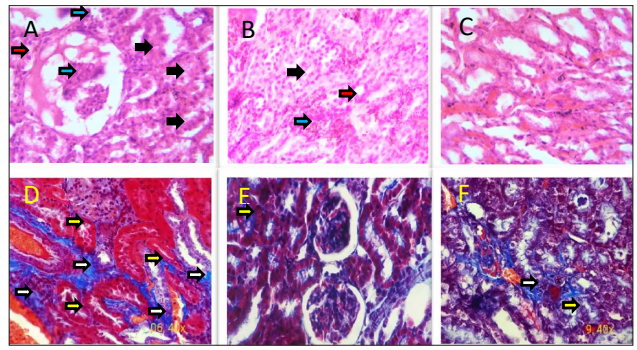
Table 3. Effect of luteolin on MDA, MPO and Hydroxyproline (HP) in STZ induced diabetic rats

Groups and Treatment	MDA nmol/mg of protein	MPO uM/mg of protein	HP ug/mg of protein
Diabetic IR Control + Vehicle	78.52±3.5#	56.9±2.2#	8.6±0.37#
Diabetic Sham + No I/R + Vehicle	42.8±2.1	42.2±1.1	4.6±0.04
Diabetic +I/R LT 50 mg/kg p.o	18.2±1.5*	24.4±1.1*	4.5±0.29*

Note: Values are expressed as mean ± S.E.M. (n=6), using one-way ANOVA followed by Dunnett's test. #p<0.01 when the diabetic IR control group is compared with the diabetic sham group, *p<0.01 when the LT treated group is compared with the diabetic IR control group (n=6)

Effect of luteolin on morphological changes in kidney in the STZ-induced diabetic rats

Kidney tissue was stained with HE to assess gross pathologic abnormalities and with MT staining to assess fibrosis. Figure 2 shows the effect of drugs on ischemic kidney damage in diabetic rats before the 2-week drug pretreatment on HE and MT-stained kidney tissue. Herein, A: diabetic IR control (vehicle); B: sham group (vehicle); C: LT 50 mg/kg p.o. group in HE staining: Changes in vascular anomalies (red arrow), tubular necrosis and degeneration (black arrow) and neutrophil infiltration (blue arrow) in the kidneys; D: diabetic IR control (vehicle); E: sham group (vehicle); F: LT 50 mg/kg p.o group in MT staining: changes in collagen deposition (white arrow) and tubular degeneration (yellow arrow) in the kidneys. Histological examination revealed necrosis and congestion in the glomeruli, inflammatory WBC infiltration, glomerular hyperplasia, changes in the urinary tract and dilation of tubules of renal tissue, all of which showed moderate to severe significant alteration in the diabetes IR group as compared to the sham group (p <0.01). These differences were significantly reduced in the pre-treated group that received LT (50 mg/kg) before ischemia in HE-staining (p <0.01). The LT group has minor congestion. On MT staining, the diabetes IR control group showed moderate to severe collagen deposition and tubular production as compared to the sham group, which showed minor changes, while the pre-treated LT group showed no to rare collagen deposition and tubular degeneration (p <0.01).



A: diabetic + I/R control; B: sham group; C: LT 50 mg/kg p.o. + diabetics + I/R in HE staining: to study changes in vascular anomalies (red arrow), tubular necrosis and degeneration (black arrow), and neutrophil infiltration (blue arrow) in the kidneys. D: diabetic + I/R control; E: sham group; F: LT 50 mg/kg p.o. + diabetics + I/R in MT staining: to study changes in collagen deposition (white arrow) and tubular degeneration (yellow arrow) in the kidneys

H&E Staining (A, B, C) (100×); Masson Trichome Staining (D, E, F) (40×)

Figure 2. The effect of LT on HE and MT stained kidney tissue

DISCUSSION

Studies have shown that diabetes exacerbates renal IR-induced AKI. However, the data linking the early stages of diabetes to IR-induced AKI is sparse. STZ-induced diabetes and acute kidney IR models [6,47] have revealed that early diabetes can exacerbate IR-induced AKI by worsening glomerular and tubular damage, as well as oxidative, proinflammatory and profibrotic processes. Pre-treatment with antioxidants may lessen renal I/R damage [6]. As both HDAC-2 and MMPs play important roles in the progression of diabetes, we investigated the effects of LT, a flavonoid molecule, on inhibition of HDAC-2 and MMPs. We employed a variety of biochemical parameters, oxidative profiles, pro-inflammatory mediators and pathological study to assess the therapeutic effects of LT on renal function.

According to recent investigation, oxidative stress damages endothelium glycocalyx by increasing HDAC activity, leading to increased MMP production and decreased TIMP production by cleaving syndecan and proteoglycans [49]. The transmembrane proteins claudin-2 and 5 are present in glomerular tight junctions, and proximal convoluted tubules are susceptible to the action of MMP-2 and MMP-9, which promote proteolytic degradation of tight

junctions. ROS and hyperglycemia are known to exacerbate oxidative stress, and MMP activity is promoted in the early stages of DN, which compromises the integrity of claudins by cleaving the extracellular domain [50]. In a type 1 DN mouse model, early treatment with an MMP-2 and MMP-9 inhibitor can repair glomerular glycocalyx architecture by retaining syndecan-4 [51]. Collagen-IV is the main type of collagen in the ECM, and GBM is destroyed by MMP, leading to abnormal ECM deposition and degradation [52]. It is evident from the results of previous research that diabetic mice with renal ischemia have higher levels of collagen [47,48]. In our study, the expression of MMP-2, MMP-9 and HDAC-2 increased significantly in the diabetic rats with renal ischemia, while pre-treatment with LT for two weeks before renal ischemia reduced this expression in diabetic rats. This suggests a potential role for metallozymes in breaking down glomeruli and tubule normal structure. Pre-treatment with LT may also reduce collagen invasion, suggesting early targeting has a protective effect in AKI with diabetes by inhibiting metallozymes (MMPs and HDAC-2).

The streptozotocin-induced diabetic rat model is a key research tool in investigations of early diabetic kidney damage, notably that in the langerhans islets [53]. The hypoglycemic and antioxidant action of LT reduce lipid synthesis and glycated hemoglobin, and preserve the pancreas, which aids in insulin secretion [54,55]. The hypoglycemic effects of LT may be due to its ability to rejuvenate or repair surviving cells, thereby increasing insulin release. Chronic hyperglycemia reduces body weight in rats through excessive breakdown of tissue proteins [47]. We investigated the effect of LT pre-treatment on renal system impairment in diabetic rats with AKI caused by ischemia/reperfusion damage. Results showed that LT pre-treatment significantly reduced hyperglycemia in diabetic rats. Additionally, LT therapy improved body weight and prevented muscle tissue damage caused by hyperglycemia-induced muscle tissue damage.

The expression of MMP-2 and MMP-9 in the blood and urine is linked to the clinical endpoint of acute kidney injury (AKI), and these levels seem to have increased faster in comparison to those of serum creatinine [56]. In our work, the control group of diabetic I/R rats showed increased serum creatinine and BUN levels, while creatinine clearance decreased after injury, indicating progressive nephrotoxicity in hyperglycemic rats [7,23,47]. In the present investigation, elevated kidney parameters were linked to decreased serum total protein and albumin levels - thereby affecting diabetic nephropathy. Pre-treatment with LT in diabetic rats showed reduced creatinine and BUN levels, suggesting improved kidney function. LT restored kidney function by increasing serum total protein and albumin levels.

Uric acid exacerbates ischemia-reperfusion damage through the ROS and inflammatory cascade pathways in the heart [56]. Additionally, it activates inflammatory and profibrotic cytokines, which contribute to the progression of DN in people with type 1 diabetes [57,58]. Hypoxanthine builds up as a result of ischemia-reperfusion injury, where it is transformed into xanthine and uric acid in the presence of oxygen during reperfusion, resulting in the generation of superoxide and free radicals that cause more damage [4]. In other work, we observed a substantial increase in serum uric acid in diabetic rats with renal I/R damage that was reduced by pre-I/R treatment with LT. Previous research has shown that LT improved renal function in I/R rats, by lowering oxidative stress [36].

Oxidative stress triggers inflammation, leading to reactive oxygen species (ROS) production and leukocyte activation, ultimately causing more oxidative stress [59]. During ischemia, the production of free radicals occurs, resulting in damage to the organ. Reperfusion also exacerbates the damage through various mechanisms such as neutrophil infiltration, cytokine activation, cellular apoptosis, necrosis and microvascular damage [4,60].

Hyperglycemia, which causes renal dysfunction due to ROS bursts, is the main factor in developing DN. In the present study, diabetic rats exposed to renal I/R damage increased MDA, MPO and hydroxyproline activity in the renal homogenate, however, treatment with LT for two weeks before I/R showed significant restoration of MDA, MPO and HP activity [29,36,60]. LT has been shown to reduce oxidative stress and the inflammatory index and restore kidney function in diabetic rats, suggesting that its antioxidant and anti-inflammatory properties may contribute to its renoprotective effect against diabetic nephropathy. It should be noted that a previous study found that pre-treatment with LT (100 mg/kg) for 7 days before renal ischemia in Swiss albino mice significantly decreased MDA, SOD, CAT, glutathione, TNF-α, IL-6 and IL-1 in kidney tissues. Moreover, LT also decreased pro-apoptotic protein Bax and increased anti-apoptotic protein Bcl-2, while decreasing caspase-3 production, reducing apoptosis in kidney tissue [37]. Our results indicate that LT has antioxidant, anti-inflammatory and anti-fibrotic properties as it suppresses expression of renal MDA, MPO and HP – and inhibits metalloenzymes in diabetes-related AKI rats.

Several studies in the past have demonstrated that diabetic rats have a high susceptibility to renal ischemia/reperfusion injury. Accordingly, after 30 minutes of I/R damage, diabetic rats had irreversible progressive kidney damage that included tubular shrinkage, inflammation and interstitial fibrosis [40]. The main component of GBM is collagen IV, and it provides stability and shape to podocytes. The fibrotic process associated with IR-induced AKI in early-stage diabetes was evident from increased collagen staining in an ischemic animal. These findings were confirmed by Masson's trichrome analysis, as total collagen volume increased in the kidney IR group [47,48]. Our histopathological findings showed renal cell damage in diabetic rats in response to ischemic injury. More than 50% of the morphological changes were related to vascular changes, tubular necrosis and neutrophilic infiltration which were evident under light microscopic examination in HE staining. Similarly, collagen deposition and tubular degeneration are the confirmatory parameters for fibrosis in MT staining. In our study, the glomerular architecture was well preserved in the two-week pre-treated LT (50 mg/kg p.o.) group and also abnormalities in the urinary tract, glomerular hyperplasia, inflammatory cell infiltration and tubular edema of the renal tissue were either absent or insignificantly seen. The kidneys of rats with STZ-induced diabetic ischemia,

in contrast, showed significant collagen deposition and tubular degeneration. Moreover, pre-treatment with LT before ischemia reduced fibrosis, supporting the structural evidence for the renoprotective effect of LT. Our studies are in line with previous studies that LT exerts the renoprotective effect by blocking and attenuating a variety of signaling pathways [36,37].

The histological damage we saw correlates with the production of oxidative stress because of the increased expression of MDA. In addition, a rise in MPO activity leads to pro-inflammatory mediators due to a rise in the MPO of renal homogenate from diabetic rats. AKI in diabetes is known to increase serum creatinine and BUN levels, which are markers of glomerular and tubular health. Pre-treatment with LT reduced elevated serum Cr and BUN in I/R, indicating improved renal function. Our results also showed increased expression of MMP-2, MMP-9, and HDAC-2 in renal I/R injury in diabetic rats, which are in line with previous studies [7,23,47].

To summarise, our data shows that early targeting of MMP-2, MMP-9, and HDAC-2 with LT can preserve glomerular and tubular architecture, arresting disease development in diabetic rats with renal ischemia. A two-week pre-treatment with LT (50 mg/kg) improved glucose homeostasis and body weight while reducing blood creatinine, BUN and uric acid levels. LT pre-treatment increased total protein and albumin, reduced oxidative damage and restored MPO and hydroxyproline expression. Thus, LT may halt the progression of kidney damage in renal ischemia-diabetic situations.

CONCLUSION

Our results imply that antioxidant pre-treatment confers renoprotection by allowing early targeting of metalloenzymes that damage the fundamental structural components of the kidneys. Pre-treatment with LT lowers oxidative stress, pro-inflammatory mediators, fibrosis and renal biochemical parameters, which improve renal function. Pre-treatment with LT in diabetics with AKI can reverse the progression of kidney damage and is a safe and affordable treatment option for diabetic nephropathy. More research is needed, however, to determine how LT affects specific signalling pathways in diabetic nephropathy.

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REFERENCES

1. Bonventre JV, Weinberg JM. Recent advances in the pathophysiology of ischemic acute renal failure. *JASN.* 2003;14:2199-210.
2. Lejay A, Fang F, John R, Van JAD, Barr M, Thaveau F, et al. Ischemia reperfusion injury, ischemic conditioning and diabetes mellitus. *JMCC.* 2016;91:11-22.
3. Zhang Y, Hu F, Wen J, Wei X, Zeng Y, Sun Y, et al. Effects of sevoflurane on NF-κB and TNF-α expression in renal ischemia–reperfusion diabetic rats. *IR.* 2017;66(10):901-10.
4. Chan KL. Role of Nitric Oxide in ischemia and reperfusion injury. *Curr Med Chem Anti-Inflammatory & Anti-Allergy Agents.* 2002;1;1-13.
5. Sutton TA, Kelly KJ, Mang HE, Plotkin Z, Sandoval RM, Dagher PC. Minocycline reduces renal microvascular leakage in a rat model of ischemic renal injury. *Am J Physiol Renal Physiol*. 2005;288:91-7.
6. Gong DJ, Wang L, Yang YY, Zhang JJ, Liu XH. Diabetes aggravates renal ischemia and reperfusion injury in rats by exacerbating oxidative stress, inflammation, and apoptosis. *Ren Fail.* 2019;41(1): 750-61.
7. Kunugi S, Shimizu A, Kuwahara N, Du X, Takahashi M, Terasaki Y, et al. Inhibition of matrix metalloproteinases reduces ischemia-reperfusion acute kidney injury. *Lab Invest.* 2011;91(2):170-80.
8. Zakiyanov O, Kalousová M, Zima T, Tesař V. Matrix metalloproteinases in renal diseases: a critical appraisal. Kidney *Blood Press Res.* 2019;44:298-330.
9. Mathalone N, Lahat N, Rahat MA, Bahar-Shany K, Oron Y, Geyer O. The involvement of matrix metalloproteinases 2 and 9 in rat retinal ischemia. *Graefe's Arch Clin Exp Ophthalmol.* 2007;245(5):725-32.
10. Muhs BE, Plitas G, Delgado Y, Ianus I, Shaw JP, Adelman MA, et al. Temporal expression and activation of matrix metalloproteinases-2, -9, and membrane type 1 – Matrix metalloproteinase following acute hindlimb ischemia. *J Surg Res.* 2003;111(1):8-15.
11. Roach DM, Fitridge RA, Laws PE, Millard SH, Varelias A, Cowled PA. Up-regulation of MMP-2 and MMP-9 leads to degradation of type IV collagen during skeletal muscle reperfusion injury; protection by the MMP inhibitor, doxycycline. *Eur J Vasc Endovasc Surg.* 2002;23(3):260-9.
12. Cheung PY, Sawicki G, Wozniak M, Wang W, Radomski MW, Schulz R. Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. *Circulation*. 2000;101(15):1833-9.
13. Wang W, Schulze CJ, Suarez-Pinzon WL, Dyck JRB, Sawicki G, Schulz R. Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. *Circulation*. 2002;106(12):1543-9.
14. Basile DP, Fredrich K, Weihrauch D, Hattan N, Chilian WM, Angiostatin WMC. Angiostatin and matrix metalloprotease expression following ischemic acute renal failure. *J Physiol Renal Physiol.* 2004;286:893-902.
15. Sutton TA, Kelly KJ, Mang HE, Plotkin Z, Sandoval RM, Dagher PC. Minocycline reduces renal microvascular leakage in a rat model of ischemic renal injury. *Am J Physiol Renal Physiol.* 2005;288:91-7.
16. Melin J, Hellberg O, Larsson E, Zezina L, Fellström BC. Protective effect of insulin on ischemic renal injury in diabetes mellitus. *Kidney Int*. 2002;61(4):1383-92.
17. Cheng S, Pollock AS, Mahimkar R, Olson JL, Lovett DH, Cheng S, et al. Matrix metalloproteinase 2 and basement membrane integrity: a unifying mechanism for progressive renal injury. *FASEB J.* 2006;20(11):1898-900.
18. Dejonckheere E, Vandenbroucke RE, Libert C. Matrix metalloproteinases as drug targets in ischemia/reperfusion injury. *Drug Discovery Today.* 2011;16:762-78.
19. Dong Y, Zhao H, Man J, Fu S, Yang L. MMP-9-mediated regulation of hypoxia-reperfusion injury-related neutrophil inflammation in an in vitro proximal tubular cell model. *Ren Fail.* 2021;43(1):900-10.
20. Noh H, Oh EY, Seo JY, Yu MR, Kim YO, Ha H, et al. Histone deacetylase-2 is a key regulator of diabetes-and transforming growth factor-1-induced renal injury. *Am J Physiol Renal Physiol.* 2009;297:729-39.
21. Granger A, Abdullah I, Huebner F, Stout A, Wang T, Huebner T, et al. Histone deacetylase inhibition reduces myocardial ischemia-reperfusion injury in mice. *FASEB J.* 2008;22(10):3549-60.
22. Fan J, Alsarraf O, Dahrouj M, Platt KA, Chou CJ, Rice DS, et al. Inhibition of HDAC2 protects the retina from ischemic injury. *Invest Ophthalmol Vis Sci.* 2013;54(6):4072-80.
23. Aufhauser DD, Hernandez P, Concors SJ, O'Brien C, Wang Z, Murken DR, et al. HDAC2 targeting stabilizes the CoREST complex in renal tubular cells and protects against renal ischemia/reperfusion injury. *Sci Rep.* 2021;11(1).
24. Cosentino CC, Skrypnyk NI, Brilli LL, Chiba T, Novitskaya T, Woods C, et al. Histone deacetylase inhibitor enhances recovery after AKI. *JASN.* 2013;24(6):943-53.
25. Du Y, Tang G, Yuan W. Suppression of HDAC2 by sodium butyrate alleviates apoptosis of kidney cells in db/db mice and HG-induced NRK-52E cells. *Int J Mol Med.* 2020;45(1):210-22.

26. Ma X, Wang Q. short-chain fatty acids attenuate renal fibrosis and enhance autophagy of renal tubular cells in diabetic mice through the HDAC2/ULK1 axis. *Endocrinol Metab.* 2022;37(3):432-43.
27. Xiong C, Wu Q, Fang M, Li H, Chen B, Chi T. Protective effects of luteolin on nephrotoxicity induced by long-term hyperglycaemia in rats. *J Int Med Res*. 2020;48(4).
28. Zhang M, He L, Liu J, Zhou L. Luteolin attenuates diabetic nephropathy through suppressing inflammatory response and oxidative stress by inhibiting STAT3 pathway. *Expe Clin Endocrinol Diabetes*. 2021;129(10):729-39.
29. Wang GG, Lu XH, Li W, Zhao X, Zhang C. Protective effects of luteolin on diabetic nephropathy in STZ-induced diabetic rats. *Evid Based Complement Alternat Med*. 2011;2011:323171.
30. Aziz N, Kim MY, Cho JY. Anti-inflammatory effects of luteolin: A review of in vitro, in vivo, and in silico studies. *J Ethnopharmacol*. 2018;225:342-58.
31. Lv J, Zhou D, Wang Y, Sun W, Zhang C, Xu J, et al. Effects of luteolin on treatment of psoriasis by repressing HSP90. *Int Immunopharmacol*. 2020;79.
32. López-Lázaro M. Distribution and biological activities of the flavonoid luteolin. *Mini Rev Med Chem*. 2009;9(1):31-59.
33. Arslan BY, Arslan F, Erkalp K, Alagöl A, Sevdi MS, Yıldız G, et al. Luteolin ameliorates colistin-induced nephrotoxicity in the rat models. *Ren Fail*. 2016;38(10):1735-40.
34. Domitrović R, Cvijanović O, Pugel EP, Zagorac GB, Mahmutefendić H, Škoda M. Luteolin ameliorates cisplatin-induced nephrotoxicity in mice through inhibition of platinum accumulation, inflammation and apoptosis in the kidney. *Toxicology*. 2013;310:115-23.
35. Xin S bin, Yan H, Ma J, Sun Q, Shen L. Protective effects of luteolin on lipopolysaccharide-induced acute renal injury in mice. MSM. 2016;22:5173-80.
36. Hong X, Zhao X, Wang G, Zhang Z, Pei H, Liu Z. Luteolin treatment protects against renal ischemia-reperfusion injury in rats. *Mediators Inflamm*. 2017;2017.
37. Liu Y, Shi B, Li Y, Zhang H. 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. *MSM*. 2017;23:5720-7.
38. Bonino B, Leoncini G, De Cosmo S, Greco E, Russo GT, Giandalia A, et al. Antihypertensive treatment in diabetic kidney disease: The need for a patient-centered approach. *Medicina*. 2019;55(7):382.
39. El Mouhayyar C, Riachy R, Khalil AB, Eid A, Azar S. Inhibitors in diabetes and microvascular complications: A review. *Int J Endocrinol*. 2020;2020:1762164.
40. Ozbilgin S, Ozkardesler S, Akan M, Boztas N, Ozbilgin M, Ergur BU, et al. Renal ischemia/reperfusion injury in diabetic rats: The role of local ischemic preconditioning. *Biomed Res Int*. 2016;2016:8580475.
41. Chowdhury S, Ghosh S, Das AK, Sil PC. Ferulic acid protects hyperglycemia-induced kidney damage by regulating oxidative insult, inflammation and autophagy. *Front Pharmacol*. 2019;10(FEB).
42. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem*. 1979;95(2):351-8.
43. Suzuki K, Ota H, Sasagawa S, Sakatani T, Fujikura T. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. *Anal Biochem*. 1983;132(2):345-52.
44. Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys*. 1961;93(2):440-7.
45. Daude RB, Shah JS. Protective effect of alpha-cyperone in renal ischemia-reperfusion induced acute kidney injury by modulation of metalloenzyme expression. *Eur Chem Bull*. 2023;12:5629-43.
46. Pourghasem M, Shafi H, Babazadeh Z, Zahra F. Histological changes of kidney in diabetic nephropathy Caspian. *Caspian J Intern Med*. 2015;6.
47. de Ponte MC, Cardoso VG, Gonçalves GL, Costa-Pessoa JM, Oliveira-Souza M. Early type 1 diabetes aggravates renal ischemia/reperfusion-induced acute kidney injury. *Sci Rep*. 2021;11(1).
48. Naylor RW, Morais MRPT, Lennon R. Complexities of the glomerular basement membrane. *Nat Rev Nephrol*. 2021;17:112-27.
49. Ali MM, Mahmoud AM, Le Master E, Levitan I, Phillips SA. Role of matrix metalloproteinases and histone deacetylase in oxidative stress-induced degradation of the endothelial glycocalyx. *Am J Physiol Heart Circ Physiol*. 2019;316:647-63.
50. Rosas-Martínez L, Rodríguez-Muñoz R, Namorado-Tonix M del C, Missirlis F, del Valle-Mondragón L, Sánchez-Mendoza A, et al. Hyperglycemic levels in early stage of diabetic nephropathy affect differentially renal expression of claudins-2 and -5 by oxidative stress. *Life Sci*. 2021;268.
51. Ramnath RD, Butler MJ, Newman G, Desideri S, Russell A, Lay AC, et al. Blocking matrix metalloproteinase-mediated syndecan-4 shedding restores the endothelial glycocalyx and glomerular filtration barrier function in early diabetic kidney disease. *Kidney Int*. 2020;97(5):951-65.
52. Kumar Bhatt L, Addepalli V. Minocycline with aspirin: An approach to attenuate diabetic nephropathy in rats. *Ren Fail*. 2011;33(1):72-8.
53. Tong Y, Liu S, Gong R, Zhong L, Duan X, Zhu Y. Ethyl vanillin protects against Kidney injury in diabetic nephropathy by inhibiting oxidative stress and apoptosis. *Oxid Med Cell Longev*. 2019;2019.
54. Zang Y, Igarashi K, Li YL. Anti-diabetic effects of luteolin and luteolin-7-O-glucoside on KK-Ay mice. *Biosci Biotechnol Biochem*. 2016;80(8):1580-6.
55. Zhang Y, Tian XQ, Song XX, Ge JP, Xu YC. Luteolin protect against diabetic cardiomyopathy in rat model via regulating the AKT/GSK-3β signalling pathway. *Biomed Res*. 2017;28(3).
56. Shen SC, He F, Cheng C, Xu BL, Sheng JL. Uric acid aggravates myocardial ischemia –reperfusion injury via ROS/NLRP3 pyroptosis pathway. *Biomed Pharmacother*. 2021;133:110990.
57. Jalal DI, Maahs DM, Hovind P, Nakagawa T. Uric acid as a mediator of diabetic nephropathy. *Semin Nephrol*. 2011;31(5):459-65.
58. Hovind P, Rossing P, Tarnow L, Johnson RJ, Parving HH. Serum uric acid as a predictor for development of diabetic nephropathy in type 1 diabetes: An inception cohort study. *Diabetes*. 2009;58(7):1668-71.
59. Ruiz S, Pergola PE, Zager RA, Vaziri ND. Targeting the transcription factor Nrf2 to ameliorate oxidative stress and inflammation in chronic kidney disease. *Kidney Int*. 2013;83:1029-41.
60. Kalbolandi SM, Gorji AV, Babaahmadi-Rezaei H, Mansouri E. Luteolin confers renoprotection against ischemia – reperfusion injury via involving Nrf2 pathway and regulating miR320. *Mol Biol Rep*. 2019;46(4):4039-47.