Resveratrol protects human renal proximal tubular cells from high glucose and hypoxia/reoxygenation induced injury via inhibiting p38-MAPK and thioredoxin-interacting protein (TXNIP) pathways

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Abstract: Patients with diabetes are more susceptible to acute kidney injury (AKI), but the underlying mechanisms and therapeutic strategies remain unclear. Much evidence suggests that tubular cells apoptosis induced by oxidative stress and inflammatory reaction is involved in diabetes and AKI. The aim of the present study was to determine the mechanism of hyperglycemia related susceptibility of ischemic AKI and to examine whether resveratrol (RES) could attenuate cell injury induced by high glucose and hypoxia/reoxygenation (HH/R) via p38-MAPK/TXNIP signaling. Human renal proximal tubular cells (HK-2 cells), cultured in high glucose (HG: 30 mM) for 72 hours with or without RES (50 μM) pretreatment, were subjected to hypoxia/reoxygenation (H/R) in the presence or absence of TXNIP and p38-MAPK achieved by gene knock-down with VDUP-1 (TXNIP) siRNA and SB203580, as a selective inhibitor of p38-MAPK. The protein expression of p-p38-MAPK and TXNIP in HK-2 cells exposed to HG and H/R insult were significantly increased, accompanied with severe cell injury, oxidative stress and inflammatory reaction, and elevated the level of cell apoptosis. All these changes were attenuated or reversed by RES treatment, and similar effects were shown in the treatment group by inhibition of TXNIP with VDUP-1 (TXNIP) siRNA or with SB203580. Our results suggest that the protective effects of RES may be achieved through inhibition of p38-MAPK activation and expression of TXINP in HK-2 cells exposed to HG and H/R insult. Thus, the RES could be a candidate for inhibiting ischemia-reperfusion induced AKI in diabetes.

Keywords: Resveratrol, p38-MAPK/TXNIP, oxidative stress, inflammatory mediator, high-glucose, hypoxia-oxygenation injury

Introduction

Hyperglycemia is the causative factor in the sensitivity of organs to damages including kidney [1]. The foremost causes of AKI include ischemia-reperfusion injury (I/RI) and hypoxia [2]. Many reports indicate that proximal tubular cells (HK-2 cell) are the most sensitive cells to renal I/RI [3, 4]. So, protecting proximal tubular cells from renal I/RI has been considered a meaningful strategy for prevention and treatment [5].

Ischemia results in hypoxia and then reoxygenation from reperfusion are involved in excessive production of reactive oxygen species (ROS) to initiate a cascade of deleterious cellular responses [6]. Diabetes has been identified as an independent risk factor for AKI, this is also related to inflammatory response and cell apoptosis [7, 8]. However, the underlying mechanisms that hyperglycemia-related acceleration renal cell damage after AKI and therapeutic strategy against AKI in diabetic patients are not clear.

The thioredoxin (TRX) is a cellular anti-oxidant [9], whereas TRX-interacting protein (TXNIP) is an intracellular endogenous inhibitor of TRX [10]. It was reported that HK-2 cells exposed to high glucose could up-regulate TXNIP expression [11], and promote HK-2 cells apoptosis [12]. Activation of p38 mitogen-activated protein kinase (p38-MAPK) pathway is involved in
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human renal disease, including diabetic nephropathy and acute renal failure [13]. Previous studies have demonstrated that p38-MAPK pathway contributes to the HG-induced upregulation of TXNIP expression and further increases intracellular ROS level [14, 15]. However, the influence of p38-MAPK on TXNIP expression in high glucose combined H/R-injury in HK-2 cells is unknown.

Resveratrol (trans-3,4,5-trihydroxystilbene, RES) is a natural polyphenol, present in grapes and red wine [16]. Pharmacologic benefits of RES include anti-oxidant [17], anti-inflammatory [18]. Moreover, the beneficial effects of RES have been showed in diabetic animal models and patients with diabetes [19], and in renal diseases including ischemic/reperfusion injury [20] and diabetic nephropathy [21]. However, the protective effects of RES in diabetic kidney to ischemia-reperfusion injury have not been thoroughly investigated. Therefore, this study aims to investigate the protective effects of RES in HK-2 cells against HG combined H/R (HH/R) induced injury. We hypothesized that RES could suppress inflammatory response and oxidative stress in HK-2 cells during HH/R. Furthermore, we examined whether RES could attenuate HH/R-induced injury in HK-2 cells through inhibiting p38-MAPK activation and TXNIP expression.

Materials and methods

Cell culture and treatments

HK-2 cells (obtained from ATCC, American Type Culture Collection, Manassas, VA) were cultured in MEM medium (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, USA), 100 U/ml penicillin, and 100 ug/ml streptomycin in a 95% air, 5% CO₂ atmosphere. The cells were pretreated with p38-MAPK inhibitor SB 203580 (20 μM, Cell Signaling Technology, USA) for 30 min [11] or transfected with TXNIP-siRNA for 48 hours then exposed to different glucose concentrations from 5.6 mM (NG) and 30 mM (HG) for 72 hours with or without 50 μM RES (Sigma-Aldrich, St. Louis, MO, USA) treated [21].

Cell viability measurement

CCK-8 was used to measure cell viability according to the manufacturer’s instructions. Briefly, HK-2 cells were seeded in a 96-well microplate at 1×10⁵ cells/well and then pre-treated with RES at a series of concentrations (0, 12.5, 25, 50, and 100 μM) for 72 hours culture. Subsequently, CCK-8 solution (10 μL/well) was added to the wells, the plate was incubated at 37°C for 4 hours, and the absorbance was determined with a microplate reader (Tecan, Mannedorf, Switzerland) at a wavelength of 450 nm.

RNAi transfections

Silencing gene expression was achieved using specific small interfering RNA (siRNA) against TXNIP (Santa Cruz Biotechnology, Santa Cruz, USA). Cells seeded (2×10⁵ per well) in six-well plates were transfected with siRNA using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer’s instructions. Scrambled-siRAN was used as control. The cells were used for further experiments 48 hours after transfection.

Measurement of malondialdehyde (MDA), superoxide dismutase (SOD), reactive oxygen species (ROS)

Intracellular formation of ROS was detected using the fluorescence probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Jiansheng Biotech, Nanjing, China). The measurement of intracellular ROS was performed using a flow cytometer. The malondialdehyde (MDA) levels, superoxide dismutase (SOD) were detected using commercially-available kits (Jiansheng
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**Measurement of TNF-α, IL-1β, LDH level**

The quantification of TNF-α and IL-1β was carried out by using the TNF-α and IL-1β commercial enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer’s instructions (Elabscience Biotechnology Co., Ltd, Wuhan, China). LDH content was measured by LDH Cytotoxicity Assay Kit (Iancheng Biotech, Nanjing, China).

**Immunofluorescence staining**

Immunofluorescence staining was carried out when the HK-2 cells reached 80% confluence. After each treatment mentioned above, the cells were fixed in 4% paraformaldehyde-PBS for 15 minutes and then immersed in 0.5% Triton X-100 (Beyotime, Shanghai, China) for 20 min at room temperature. Then the cells were blocked with normal goat serum (Boster, Wuhan, China) for 30 minutes at room temperature. Thereafter, the fixed and permeabilized HK-2 cells were incubated with the primary anti-p-p38-MAPK (1:100) and anti-TXNIP (1:50) antibody at 4°C overnight, followed by another incubation with the secondary antibody (Boster, Wuhan, China) for 1 hour at 20-37°C. Cellular nucleus were stained with DAPI (Beyotime, Shanghai, China). Under 400 (200)× magnification, images were taken by fluorescence microscope (Olympus, Japan).

**Apoptosis assay**

The percentage of apoptosis was evaluated by using an Annexin V-APC/7-AAD detection Kit (NANJING KEYGEN BIOTECH, Nanjing, China) according to the manufacturer’s instructions. Samples were assayed by flow cytometry with the FACScan system (BD Biosciences). Apoptotic cells were defined as the cells situated in the right two quadrants of each plot and the percentages were determined by flow cytometry.

**Western blot analysis**

The expressions of TXNIP, p38-MAPK and p-p38-MAPK were measured using Western blot. Protein content was determined with BCA protein assay and protein samples were separated by electrophoresis on SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% milk and incubated overnight with the appropriate primary antibodies respectively (Anti-TXNIP, 1:500, Abcam, USA. Anti-p38 and anti-p-p38, 1:1000, CST, USA), followed by incubation with the corresponding secondary antibodies. The blots were visualized with ECL-plus reagent. GAPDH was used as the internal loading control.

**Statistical analysis**

Data are expressed as the means ± SE (standard error) in each group. Experimental results were analyzed using a one-way ANOVA followed by Tukey’s test for multiple comparisons between the means of each group. All statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA, USA). A value of P<0.05 was considered to indicate a statistically significant difference.
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Figure 2. RES restrained oxidative stress injury in HK-2 cells with the model of HG combined H/R (HH/R). HK-2 cells incubated with normal glucose (5.6 mM, NG), high glucose (30 mM, HG), and HG plus RES (50 μM) for 72 hours. After hypoxia 4 hours and re-oxygenation 2 hours (H/R), intracellular ROS (A) was detected by flow cytometry, MDA (B) contents and SOD (C) contents were detected by commercially-available assay kits. Data are presented as the means ± SE (n=5). #P<0.05 vs NG group, ★P<0.05 vs HG group, &P<0.05 vs NH/R group, ▲P<0.05 vs HH/R group. NG: normoglycemic (5.6 mM) and HG: hyperglycemic (30 mM). NH/R: hypoxia/re-oxygenation under NG condition and HH/R: hypoxia/re-oxygenation under HG condition. HH/R-RES: HH/R pretreated by RES (50 μM) for 72 hours.

Figure 3. RES treatment alleviate TNF-α and IL-1β release and apoptosis rate. Cell apoptosis (C) was analyzed by flow cytometry, apoptotic cells were defined as the cells situated in the right two quadrants of each plot and the percentages were determined by flow cytometry. The level of TNF-α (A) and IL-1β (B) assayed by ELISA. Data are presented as the means ± SE (n=5). #P<0.05 vs NG group, ★P<0.05 vs HG group, &P<0.05 vs NH/R group, ▲P<0.05 vs HH/R group. NG: normoglycemic (5.6 mM) and HG: hyperglycemic (30 mM). NH/R: hypoxia/re-oxygenation under NG condition and HH/R: hypoxia/re-oxygenation under HG condition. HH/R-RES: HH/R pretreated by RES (50 μM) for 72 hours.
Figure 4. Effect of blockage of p38-MAPK on HG-induced H/R injury and protective of RES treatment. p38-MAPK and phosphorylation of p38-MAPK (A-C) and TXNIP (A, D) in HK-2 cells were analyzed by Western blot. The immunofluorescence staining of p-p38-MAPK and TXNIP (E, F). Data are presented as the means ± SE (n=5), *P<0.05 vs NG group, #P<0.05 vs HG group, &P<0.05 vs NH/R group, ▲P<0.05 vs HH/R group. NG: normo-glycemic (5.6 mM) and HG: hyperglycemic (30 mM). NH/R: hypoxia/re-oxygenation under NG condition and HH/R: hypoxia/re-oxygenation under HG condition. HH/R-RES: HH/R pretreated by RES (50 μM) for 72 hours and HH/R-SB203580: HH/R pretreated by SB203580 (20 μM) for 0.5 hours.
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Results

Effect of RES on cell viability and LDH release in HK-2 cells exposed to high glucose and hypoxia-oxygenation insult

As shown in Figure 1A, HK-2 cells were incubated with RES in various concentrations (12.5, 25, 50 and 100 μM) for 72 hours, the dose of RES (12.5, 25, 50 μM) did not affect the cell viability of HK-2 cells, but 100 μM of RES significantly decreased cell viability compared to the NG group. Refer to previous study, in which RES at the dose of 50 μM for 72 hours could abrogate high glucose (30 mM) induced protein synthesis in the epithelial cell, and incubated at high concentrations of RES for 48 hours, it inhibits expression of TXNIP in cancer cells [23]. We therefore selected 50 μM RES as the experimental concentration in the following studies. As shown in Figure 1B and 1C, both HG and H/R markedly increased LDH release and reduced cell viability as compared to NG group. Moreover, HH/R showed a further increase of LDH and decline in cell viability as compared to NH/R group. 50 μM of RES significantly decreased the level of LDH in HK-2 cells exposed to HH/R and prevented HH/R induced reduction of cell viability.

Effect of RES on oxidative stress in HK-2 cells exposed to HH/R

As shown in Figure 2, in comparison with NG group, HG and NH/R significantly increased ROS (Figure 2A) production and MDA (Figure 2B) levels. Moreover, HH/R showed a further rise of ROS and MDA as compared to NH/R group. In contrast, the SOD (Figure 2C) activity was significantly reduced in NH/R or HH/R group as compared to NG or HG group respectively. Moreover, HH/R further decreased the level of SOD as compared to NH/R group. Next, we examined if RES modulated HH/R-induced oxidative injury, simultaneous incubation with RES (50 μM) for 72 h markedly alleviated oxidative stress by markedly decreased ROS (Figure 2A) and MDA (Figure 2B), increased SOD (Figure 2C) than HH/R group.

Effect of RES on inflammation and cell apoptosis in HK-2 cells exposed to HH/R

As shown in Figure 3, all of inflammatory cytokines TNF-α (Figure 3A), IL-1β (Figure 3B) and the cells apoptosis rate (Figure 3C) were markedly higher under HG or H/R conditions than NG group. Moreover, HH/R showed a further rise of TNF-α and IL-1β and apoptosis rate as compared to NH/R group. Treatment with RES significantly decreased TNF-α and IL-1β level and apoptosis rate in HK-2 cells as compared with HH/R group.

Effect of RES on p38-MAPK and TXNIP expression, and blockage of p38-MAPK pathway inhibited expression of TXNIP after H/R stimulation in HG cultured HK-2 cells

As shown in Figure 4, to assay whether p38-MAPK kinase pathway affected HG plus H/R induced TXNIP expression, HK-2 cells were treated with a selective p38-MAPK inhibitor SB203580. HK-2 cells preincubated with SB203580 (20 μM) for 0.5 hours then incubated with HG for 72 hours, following hypoxia 4 and 2 hour oxygenation. The p-p38-MAPK (Figure 4A, 4C) and TXNIP (Figure 4A, 4D) expression were observably increased in HG or H/R stimulating as compared with corresponding control group respectively (NG and HG). Moreover, HH/R significantly stimulated the expression of p-p38-MAPK and TXNIP compared with NH/R group, which was markedly inhibited by SB203580 and RES than HH/R group. There was no significant difference in p38-MAPK (Figure 4A, 4B) expression among all groups. At last, using immunofluorescence staining to investigate the changes of expression about p-p38-MAPK (Figure 4E) and TXNIP (Figure 4F) in line with in vitro experiments. Mannitol had no effect on protein expression (data not shown).

Blockage of p38-MAPK pathway alleviated oxidative stress and the level of apoptosis as well as TNF-α after H/R injury in HG cultured HK-2 cells

As shown in Figure 5, to determine whether p38-MAPK pathway was involved in HG combined H/R injury, HK-2 cells incubated with HG for 72 hours and following H/R had increased intracellular ROS and MDA levels meanwhile decreased SOD content as compared with HG group (Figure 5A-C), however, this effect could be abolished by SB203580. Moreover, HH/R had obviously increased cell apoptosis in HK-2 cells than HG, however, it could be abolished by SB203580 (Figure 5D). At last, the released of
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Figure 5. Effect of blockage of p38-MAPK on the oxidative stress and the level of apoptosis as well as TNF-α with HG-induced H/R, intracellular ROS (A) was detected by flow cytometry, MDA (B) contents and SOD (C) contents were detected by commercially-available assay kits. Cell apoptosis (D) was analyzed by flow cytometry, apoptotic cells were defined as the cells situated in the right two quadrants of each plot and the percentages were determined by flow cytometry. The level of TNF-α (E) assayed by ELISA. Data are presented as the mean ± SE (n=5), ★P<0.05 vs HG group, ▲P<0.05 vs HH/R group. HG: hyperglycemic (30 mM). HH/R: hypoxia/re-oxygenation under HG condition. HH/R-SB203580: HH/R pretreated by SB203580 (20 μM) for 0.5 hours.
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TNF-α (Figure 5E) was significantly increased in HH/R group and suppressed by SB203580.

**Effect of TXNIP interference on oxidative stress level after H/R injury in HG cultured HK-2 cells**

As shown in Figure 6, to confirm whether TXNIP influences oxidative stress level, we used TXNIP siRNA to knockdown TXNIP in HK-2 cells, the expression of TXNIP protein was efficiently inhibited by transfection with TXNIP siRNA then exposure to HG for 72 hours and following H/R (Figure 6A). The HH/R-induced oxidative stress level (Figure 6B-D) were inhibited by transfection of TXNIP siRNA.

**TXNIP silencing inhibited HH/R-induced activation of p38-MAPK and released of IL-1β**

As shown in Figure 7, to investigate the modulation of p38-MAPK signal pathway by TXNIP under HH/R condition, HK-2 cells were transfected with TXNIP siRNA or scrambled siRNA. Compared with HG group, the phosphorylation level of p38-MAPK (p-p38-MAPK) (Figure 7A, 7C) and the release of IL-1β (Figure 7D) were significantly increased in HH/R group, whereas transfection of TXNIP significantly suppressed HH/R-induced p38-MAPK phosphorylation (Figure 7A, 7C) and IL-1β release (Figure 7D). There was no significant difference in p38-MAPK (Figure 7A, 7B) expression among all groups.

**Discussion**

In this study, we focused on the protective effects of resveratrol (RES) against inflammatory and oxidative injury in HK-2 cells induced by high glucose (HG) cultured and hypoxia/re-oxygenation (H/R), given the important role of the p38-MAPK/TXNIP/ROS axis in the inflammatory response and oxidative stress. The potential mechanism by which RES reduced p38-MAPK and TXNIP expression and ROS was explored by analysis of IL-1β and TNF-α production. Furthermore, RES treatment alleviated H/R-induced higher levels of apoptosis and LDH release in HG cultured HK-2 cells. Based on previous findings that RES ameliorates high glucose-induced protein synthesis in glomerular epithelial cells peaking at 30-50 μM [21]. Furthermore, the inhibition effect of RES on the expression of TXNIP at higher concentrations [23]. In the present study, RES was used at a concentration of 50 μM, which did not affect the cell viability of HK-2 cells with 72 hours culture.

It is well known that the diabetes is associated with poor prognosis of I/R including heart, kidney tissues [7, 24]. However, the possible mechanisms and therapeutic strategies are still poorly understood. Previous research
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has demonstrated that oxidative stress [25] and inflammation activation [7] involved in the acceleration of cellular damage and apoptosis which resulted in renal dysfunction. Effects of RES with anti-oxidant and anti-inflammatory were shown in diabetic animals [19]. In our present study, cultured HK-2 cells into HG for 72 hours and simulated I/R with hypoxia for 4 and 2 hours of reoxygenation (HH/R), HG induced seriously oxidative stress, release of inflammatory factors and apoptosis which in accordance with previous study [11]. Moreover, HG further promoted H/R-induced levels of apoptosis and oxidative stress (indicated by ROS, MDA and SOD), enhanced release of LDH, IL-1β and TNF-α. Furthermore, treatment of RES could significantly alleviate HH/R-induced cell injury (indicated by cell viability and LDH), suggesting a new strategy for renoprotection. However, the concrete mechanism remain further investigation.

TXNIP, also known as vitamin D₃, or thioredoxin binding protein-2, regulates the cellular redox state by binding to the redox-active cysteine residues, was induced during hypoxia in pancreatic cancer cells [26]. Meanwhile, hyperglycaemia promoted oxidative stress through inhibition of TRX activity by increased expression of TXNIP in HK-2 cells [11] and mediated IL-1β transcription in Human Adipose Tissue [27]. IL-1β is an important mediator of inflammation in I/R injury [28]. In this research, for the first time to our knowledge, high-glucose promoted H/R-induced TXNIP expression in HK-2 cells. Moreover, gene silencing of TXNIP reduced HH/R-elevated oxidative stress and IL-1β production. Thus we speculate that TXNIP may be a potential therapeutic target for ischemia AKI in diabetic kidney.

Different stimuli such as oxidative stress and hyperglycemia could activate p38-MAPK [29]. The recent study showed that p38-MAPK cause kidney I/R injury by leading redox stress and cell apoptosis [30]. Further research demonstrated that p38-MAPK mediated TNF-α production and renal tubular cell apoptosis during simulated ischemia [31]. However, there is no study examined the p38-MAPK pathway in combining H/R and HG. Here, we confirmed a previous study that either H/R or HG induced increased p38-MAPK phosphorylation [32] and following TNF-α production, cell apoptosis [31]. Data from our study also indicated that HG plus H/R significantly increased phosphorylation of p38-MAPK in cultured HK-2 cells, accompanied...
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with high levels of TNF-α and apoptosis. More importantly, TNF-α involved in increasing susceptibility with ischemia AKI of db/db mice [7]. Our result further demonstrated that blockade of p38-MAPK pathway by SB203580 in cultured HK-2 cells could not only markedly reduced p38-MAPK activation, but also decreased HH/R-induced TXNIP expression and oxidative stress level. Another study had also reported that glucose-induced ROS could upregulate TXNIP expression through p38-MAPK [14]. Su et al. found that hyperglycemia combined H/R promoted the TXNIP expression and resulted in the exacerbation of cardiomyocytes apoptosis possibly through p38-MAPK activation [32]. These observations indicated that p38-MAPK as an upstream mechanism for TXNIP expression during HG or H/R-induced injury. More interestingly, in present study, we also found that transfection with TXNIP siRNA significantly suppressed HH/R-induced activation of p38-MAPK, which damaged anti-oxidative system and then promote higher ROS level in HK-2 cells under HG combined with H/R conditions by TXNIP-TRX system.

RES had been shown to improve renal dysfunction and oxidative stress in STZ-induced diabetic rats [33] and attenuate renal ischemic injury in rats through the modulation of oxidative stress and inflammation [20]. Previous study reported that RES protected against liver and brain ischemia injury by reducing oxidative stress and expression of TXNIP [34, 35]. Other research indicated that RES-mediated inhibition of the p38-MAPK/NF-KB pathway and ROS production in HUVECs [36]. This study has shown that HH/R-induced oxidative and inflammation-mediated HK-2 cells apoptosis is ameliorated by RES via preventing p38-MAPK phosphorylation and TXNIP expression. Thus, RES may be a candidate for development of a multi-potent drug.

It should be pointed out that there are some limitations in our study. First of all, our in vitro experiments tested the effect of RES on HH/R induced TXNIP expression. However, expression or activity of TRX and cross-talk between TRX and TXNIP have not been investigated. What is more, in our study, the viability of RES in HH/R induced HK-2 cells was studied by CCK-8 assay. The treatment with 50 μM RES in HH/R induced HK-2 cells enhanced cell viability. Considering that the CCK-8 assay is a primary screening test which does not distinguish apoptosis and necrosis, further study must be carried out to clarify the specific effect of RES. Lastly, our work is limited in vitro. Additional research in diabetic rats model of renal IR/I are in progress in our laboratory.

Taken together, our data provide a molecular basis that the protective effect of RES on inflammation and oxidative injury in HK-2 cells induced by HG combined H/R through inhibition of the p-p38-MAPK and TXNIP expression. However, whether there is a direct functional interaction between p38-MAPK and TXNIP remain to be elucidated. Thus, our work provides a new molecular basis (ROS/p38-MAPK/TXNIP pathway) for RES to be a potential therapeutic for AKI in diabetic patients.

Disclosure of conflict of interest

None.

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