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Research Article

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HDAC9 promotes the susceptibility of diabetes to Contrast-induced acute kidney injury by regulating TXNIP/Trx1 pathway

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Abstract

Background Contrast-induced acute kidney injury (CIAKI) is the third most common cause of hospital-acquired AKI. Diabetes mellitus (DM) is a major risk factor for CIAKI. Nevertheless, the mechanism of its role in diabetes susceptibility to CIAKI remains unclear. This study aimed to explore the role played by HDAC9 during the susceptibility of diabetic model to CIAKI.

Methods Both in vitro and in vivo model of diabetes were induced by treating human renal tubular epithelial cells (HK-2) with high glucose (HG, 50mM) and by feeding mice with a high-fat diet (HFD) followed by intraperitoneal injection of streptozotocin (STZ), respectively. CIAKI mice models were constructed by contrast-media (iohexol), and iohexol also was treated HK-2 cells. Then, BRD-4354 (an inhibitor of HDAC9) was added into treated cells and mice. Finally, knockdown HDAC9 in HK-2 cultured with HG, and iohexol was added. The pathological changes, oxidative stress and apoptosis levels in mice kidney tissues were assessed. Meanwhile, cellular reactive oxygen species and the activity of HK-2 cells was measured. Western blot was used to determine the expression of HDAC9 and TXNIP/Trx1/P-ASK1/p38MAPK signaling pathways in cells and kidneys.

Results HDAC9 was increased in both diabetic kidney tissues and HG-induced HK-2 cells. In vitro experiment indicated that HK-2 exposed to HG attenuated further damage to apoptosis and oxidative stress by iohexol via knockdown and inhibiting HDAC9. In vivo assay revealed that BRD-4354 reduced diabetic mice sensitivity to CI-AKI. Mechanically, HDAC9 could activate TXNIP/Trx1/P-ASK1/p38MAPK signaling pathway involving in the susceptibility of diabetes to CIAKI.

Conclusion HDAC9 promotes the sensitivity of diabetes to CIAKI; and may be involved in oxidative stress and apoptosis through regulation of the TXNIP/Trx1/P-ASK1/p38MAPK pathway.

Keywords: Diabetes; Contrast-induced acute kidney injury（CIAKI）; Histone deacetylase 9 (HDAC9); Apoptosis; Oxidative Stress

Background

Diabetic kidney disease (DKD) is the main cause of kidney failure worldwide; Unlike other complications of diabetes, the incidence of diabetic kidney disease has not declined in the past 30 years (1). Diagnostic imaging techniques use radiographic contrast media (RCM), causing an acute decline in kidney function, which is known as contrast-induced acute kidney injury (CIAKI) (2, 3). CIAKI is the third reason for hospital-acquired renal failure. Its clinically definition is decline in renal function with serum creatinine peaking over 25%, or an absolute increase of 0.5 mg/dl from base line, occurring within 48 hours after contrast medium administration. Diabetes mellitus, the most common cause of chronic kidney disease, has 30% percent CIAKI occurring, especially with diabetic kidney diseases (4, 5). Additionally, diabetic patients with CIAKI have a significantly decreased survival probability compared with patients without diabetes. Contrast agents can directly cause cytotoxicity in the kidney, including renal tubular epithelial and endothelial cells (5-8), leading to mitochondrial dysfunction (9-11), apoptosis (12, 13) or necrosis (14, 15) and interstitial inflammation (10, 16-18). Meanwhile, contrast agents can alter renal hemodynamics, resulting in intrarenal vasoconstriction, causing hypoxia in the medullary lumen (8, 19), generating excessive reactive oxygen species (ROS) (19, 20) and promoting oxidative stress expression levels, which impair renal function.

Exposure of renal tubular epithelial cells to contrast agents under hyperglycemic conditions can increase the production of ROS, which in turn increases the load on the kidney and contributes to a sustained decline in renal function. Therefore, exploring the susceptibility of renal tubular epithelial cells exposed to high glucose to contrast medium-induced injury and its molecular mechanism is helpful to prevent and treat CIAKI.

Thioredoxin-interacting protein (TXNIP) is a regulatory factor associated with oxidative stress and involved in cell proliferation, differentiation and apoptosis [22]. Studies have shown that TXNIP plays an important role in the pathogenesis of diabetic nephropathy (21-25). In physiological conditions, thioredoxin (Trx) combined with TXNIP to inhibit its activity, thus resisting oxidative stress in resting cells. However, accumulated reactive oxygen species（ROS）promote TXNIP binding to the endogenous antioxidant thioredoxin (Thioredoxin , TRX) and inhibiting the antioxidant activity of the protein by exchanging disulfide bonds with Trx , thus participating in the pathogenesis of various diseases such as cancer, autoimmune diseases and diabetes (26). Thioredoxin 1 (Trx1) is localized in the cytoplasm, and reduced expression of Trx1 is a direct inhibitor or negative regulator of Apoptosis signal-regulating kinase 1 (ASK1) (27, 28), which would activate ASK1, thereby promoting its downstream substrate p38 mitogen-activated protein kinase (p38 mitogen-activated protein

kinase, p38 MAPK) phosphorylation, ultimately leading to the onset of apoptosis in renal tubular cells (29). Studies have shown that glucose can regulate the expression of TXNIP through histone acetylase, thus promoting the development of DKD(30). Histone deacetylases (HDACs) have essential roles in promoting gene transcription, which could stabilize the acetylation activities of histone acetyltransferases during chromatin remodeling. Histone deacetylase 9 (HDAC9), a member of class IIa HDAC subtype. The expression of HDAC9 mRNA was significantly increased in the renal tissue of patients with DKD, and in vitro experimental studies showed that HDAC9 expression was associated with the release of inflammatory cytokines, apoptosis of podocytes and production of reactive oxygen species (31). In addition, HDAC9 participates in lipid metabolism and arteriosclerosis progression by altering the acetylation of target histone proteins (32). However, the mechanism of its role in diabetes susceptibility to CIAKI remains unclear.

In our study, we investigated renal tubular epithelial cell injury induced by Diabetic-CIAKI, and found high glucose/diabetic conditions increase HDAC9 expression, which promotes TXNIP expression, leading to activation of ASK1 and then facilitates the substrate p38MAPK phosphorylation, eventually causing oxidative stress and apoptosis generation and promoting susceptibility of diabetic mice to CIAKI. The aim of the study was to explore the mechanism of HDAC9 in diabetes

sensitive to CIAKI and to provide new diagnostic markers and therapeutic targets for the prevention and treatment of CIAKI occurring on the basis of diabetes.

2.Methods and Materials

2.1 Mouse Models of Diabetes

C57BL/6J male mice were purchased from Hunan Slack King Experimental Animal Company (Changsha, China). For STZ-induction of diabetes, firstly, mice were fed with a high-fat diet for four weeks, then mice at 8-week of age were injected intraperitoneally with 50 mg/kg STZ for 5 consecutive days. STZ-induced mice were kept for an additional 4-5 weeks before administration of contrast medium. Blood glucose was measured 72h later to confirm hyperglycemia and the animals blood glucose level higher than 200 mg/dL were considered diabetes.

2.2 Contrast-induced acute kidney injury Model

Mice were randomly selected into 5 groups of 6 mice each: (i) control group (Con), (ii) diabetes mellitus (DM), (iii) contrast-induce acute kidney injury group (CIAK), and (iv) diabetes mellitus+ CIAKI (DM-CIAKI), (V) diabetes mellitus+ CIAKI +HDAC9I (DM-CAKI-hdi). CIAKI was triggered in mice by the method described previously. [17, 18]. In brief, after water deprivation for 16 h, for inhibition of nitric oxide synthase, mice were injected with NG-nitro-L-arginine methylester (L-NAME, 10 mg/kg i.p), followed after 15 and 30 min, respectively, by injection of

an inhibitor of prostaglandin synthesis (indomethacin,10 mg/kg, i.p) and received low-osmolar monomeric iodinated radiocontrast media iohexol (Omnipaque, 3.0g iodine/kg) through a tail vein. Controls received the same volume of saline injection. After iohexol injection, mice were allowed water and food in metabolic cages for 24h.

2.3 Cell Treatment

The human kidney cell line (HK-2), an immortalized proximal tubular epithelial cell line from the normal adult human kidney. Cells were cultured in six-well dishes and were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (Sigma) plus 1% streptomycin/penicillin (Solarbio) at 37°C in a 5% CO2 atmosphere. When the cells reached 90% confluence in culture wells, cells were randomly divided into the following groups: (i) control: the cells were treated with low serum medium; (ii) CM (iohexol): the cells were subjected to iohexol (150mgI/mL) for 4h[19]; (iii) HG (high glucose): the cells exposed to 50 mM D-glucose[14]; (iv) HG+CM（high glucose+ iohexol）: the cells were pretreated with 50 mM D-glucose for 44h and then co- subjected to iohexol (150mgI/mL) for 4h; (v) HG+CM+hdaci (HDAC inhibitor, BRD4354, a relatively selective inhibitor for HDAC9) : the cells were exposed to 50 mM D-glucose for 42h, treated with BRD-4354 (2μmol/L) for 2h, then subjected to iohexol (150mgI/mL) for 4h; (vi) si-HDAC9+ high glucose + iohexol: the cells were pretreated with HADC9 siRNA, incubated with medium

supplemented with 50mM D-glucose for 44h and, then, subjected to iohexol (150 mgI/mL) for 4h. For the HDAC9 silencing experiments in vitro, the HK-2cells were transfected with 50 nM of small interfering RNA (siRNA) specific for HDAC9 with Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) following the manufacturer's instructions for 24h. The sequence of siRNA-HDAC9 was (5′-3′) CGCAUUCUAAUUCAUGAAGAU

2.3 Cell Viability

Determination of cell viability was using Cell Counting Kit-8 (CCK-8). HK-2 cells (5×103/well) were seeded onto 96-well plates under high glucose（50mmol/L）or normal glucose (5.5mmol/L) for 48h, during which time the contrast agent and inhibitor together were exposed 4 and 6 h earlier, respectively. Next, 100 μL of fresh medium containing 10 μL of CCK-8 was added into each well for 2 h. The optical density at 450 nm was recorded using a micro-plate reader.

2.4 Immunoblotting

Western blotting was performed as described [12, 14, 20]. In brief, protein in cells and renal tissues were determined using the BCA reagent. An equal amount of protein was used SDS-PAGE to separate for electrophoresis and then transferred onto polyvinylidene fluoride (PVDF) membranes. The blots were incubated in 5% skim milk for 1h, then with primary antibodies including HDAC9 (Abcam); anti-phospho-ASK1 (abcam, Ser966); anti-

ASK1 (Abcam); anti-phospho-p38 MAPK (Cell Signaling Technology); anti-p38 MAPK (Cell Signaling Technology); anti-Trx1 (Abcam); anti-Txnip; anti-cleaved-caspase 3 (Cell Signaling Technology); anti-GRAPDH; and anti-Tubulin, at 4°C overnight, followed with HRP-conjugated secondary antibody at room temperature for 1h. The blots signal was visualized by using the enhanced chemiluminescence system.

2.5 Blood sample biochemical evaluation

Twenty-four hours after model establishment, blood samples were collected through the orbital sinus and spontaneously clotted at room temperature. Serum was centrifugated at 3,000 × *g* for 10 min at 4°C. Blood urea nitrogen (BUN) and serum creatinine (Scr) were analyzed using a commercial kit to determine renal function.

2.6 Histopathological evaluation of renal tissue

The fixed kidney tissue was dehydrated in graded series of ethanol and then embedded in paraffin. 4-μm kidney sections were cut and stained with hematoxylin and eosin (HE). We adopted published criteria as a description of each parameter that includes tubules atrophy, epithelial edema, tubular lumen dilatation, vacuolization or presence of an inflammatory cell infiltrate [21]. Tubular injury scores were calculated as following: 0, < 5%; I, 5–25%; II, 26–50%; III, 51–75%; and IV, > 75%.

2.7 Determination of apoptosis

In vivo experiments, apoptosis was determined by Terminal deoxynucleotidyl transferase dUTP nick end-labelling (TUNEL) staining according to the manufacturer's instructions. In vitro experiments, HK-2 cells were washed three times with PBS and incubated with a solution of Hoechst 33258 (2μg/mL) for 30 min at room temperature. Fluorescence images were pictured by a fluorescence microscope. In vitro HK-2 cells, Hoechst 33258 was used to observe morphology of apoptotic cells.

2.8 Analysis of intracellular ROS

In vivo mice experiment, commercial 2′,7′-dichlorodihydrofluorescein (DCFH) diacetate molecular probes (Sigma) were used to measure intracellular ROS levels. Briefly, HK-2 cells in six-well plates were cultured with 2 ml of 10 μM DCFH diacetate probes for 30 min at 37°C in the dark, then wash three times with PBS, nonfluorescent DCFH to dichlorofluorescein (DCF) can be oxidized by ROS. Furthermore, dihydroethidium (DHE) staining was used to determine the ROS in the kidney.

2.9 Oxidative Stress Markers measurement

Superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH-Px) levels in renal tissues were determined using SOD kit, MDA kit, and GSH-Px kit, respectively (Sigma, St. Louis, MO, USA). Briefly, according to the manufacturer’s instructions, kidney tissues were homogenized with detection buffer and a Thermo 3001 microplate

spectrometer was used at the corresponding absorption wavelength to obtain optical density values.

3.0 Statistical analysis

Our data were analyzed using SPSS 16.0 statistical software (IBM, Armonk, NY, USA) and expressed as means ± SEM. Multiple groups comparison were used One-way ANOVA. $p < 0.05$ was considered statistically significant. Each experiment was repeated at least three times.

3. Results

3.1 Sensitivity of STZ-induced diabetic mice to contrast-induced acute kidney injury

Contrast-induced acute kidney injury (CI-AKI) leads to worse prognosis in diabetic patients and increasing medical cost (33). To observe the phenomenon in animal models, we established CI-AKI in STZ-induced diabetic mice (DM) and non-diabetic (ND). Compared with ND mice, the levels of serum urea nitrogen (BUN) and serum creatinine (SCr) were markedly higher than DM mice. (Figure 1A-1B). In pathological HE staining of kidney tissue sections, compared with DM-CIAKI group, fewer injuries in the pathological changes of kidney were observed in the ND-CIAKI, which involves vacuolar degeneration, fragmented cells and dilatation of lumen (Figure 1C). Finally, semi-quantitative damage scoring of renal tubules showed that the tubular damage was higher in the DM-

CIAKI than in the CIAKI group. Thus, the above experimental results demonstrated that streptozotocin-induced diabetic mice were more sensitive to CIAKI.

3.2 High glucose-conditioned HK-2 cells with contrast medium (iohexol) have a synergistic effect on apoptosis

It has already been proved that high glucose (HG) stimulation could lead to cell death in human renal tubular epithelial cells(34, 35). To better know the phenomenon of diabetic kidney tissues sensitive to contrast-induced acute kidney injury, we established a model of renal epithelial cells treated with high glucose. To mimic the diabetic group, renal tubules epithelial cells were incubated in 50 mM glucose (high glucose) for 48h, and the control groups were cultured in normal glucose containing 5.5 mM glucose; followed by subjected to contrast medium (iohexol) for 4h; As shown in Figure 2A, hyperglycemia cultured with iohexol (HG+CM) group showed more apoptotic morphology and cell condensation than the normal glucose with iohexol group (CM) under the light microscope. In addition, the results of Cell Counting Kit-8 (CCK-8) assay suggested the HG+ CM groups had significantly less cell viability than the CM groups alone (Figure 2B). Next, we tested a vital mediator protein of apoptosis, the cleaved-caspase 3 in different groups. Western blot analysis showed that the use of iohexol in high glucose-conditioned HK-2 cells expressed higher level of cleaved-caspase 3, as presented in Figure 2C; Thus, conjunction of

contrast medium and high glucose significantly impaired morphology and number of HK-2 as well as increasing the number of apoptotic cells.

3.3 HDAC9 was upregulated both in both HG-cultured renal tubular epithelial cells and in diabetes mice

In vitro, a study by Liu et al (31) has been proved that high glucose significantly increased HDAC9 expression in podocytes, and further analysis indicated HDAC9 mediated inflammation and apoptosis, contributing to podocyte injury. In our experiment, we tested the expression of HDAC9 in HK-2 cells. We increased the D-glucose concentration in the medium, ranging from 5.5mM to 50mM. As shown in Figure 3A, high glucose increased HDAC9 expression; Meanwhile, as shown in Figures 3C and 3E, exposure to iohexol at high glucose in different time points also showed higher level of HDAC9 expression ($^{\&}p<0.05$ versus CM). Furthermore, compared to the control and CIAKI groups, the diabetic model showed higher expression of HDAC9 when presented by the western blotting analysis (Figures 3D and 3F, $^{**}p<0.01$ vs Con, $^{\&\&}p<0.01$ vs CIAKI).

3.4 HDAC inhibitor (HDACI) treatment mitigates oxidative stress and apoptosis in HK-2 cells co-cultured with high glucose and iohexol

To observe the effect of HDACI in HK-2 cells co-treated with iohexol and high glucose, HK-2 cells were divided into five group: (I) control ; (II) CM (iohexol, 150mgI/ml); (III) HG (50Mm glucose); (IV) HG+CM (high-

glucose+iohexol); (V) HG+CM+HDACI (high-glucose+iohexol+BRD-4354). The activity of human renal tubular epithelial cells was analyzed by CCK-8 assay. As shown in Figure 4A, the data showed a significant decrease in the activity of cells (HG+CM) in the non-inhibitor group compared to renal tubular epithelial cells (HG+CM) incubated with the pre-added inhibitor—HDACI ($^{\#}p<0.05$ vs HG+CM). This result indicated that the HDACI increased the cell activity in the HG+CM group. Also, cellular activity was similarly inhibited in the HG+CM group compared to the CM group alone（$^{\#}p<0.05$ vs HG+CM）, indicating that the addition of iohexol decreases the activity HK-2 cells exposed to high glucose. As illustrated in Figure 4B, the pro-apoptotic protein expression levels of cleaved caspase-3 was increasing in HG+CM group, whereas treatment with HDACI could strikingly reversed upregulations of cleaved caspase-3 expression. In addition, in light of HDACI-mediated beneficial effects of HG-evoked less apoptosis, we intended to determine whether HDACI altered HG-induced oxidative stress after administration of iohexol. Compared with the CM alone group, the massive production of intracellular ROS was elevated in HG+CM (Figure 4C). However, immunofluorescence (DCFDA) results confirmed this abnormality could abrogated by preconditioning with HDACI for hours in HG-induced groups. Altogether, the results indicate HDACI attenuates the apoptotic and oxidative stress of HK-2 when co-cultured with high glucose and iohexol.

3.5 HDAC9 knockdown attenuates HG-induced apoptosis and oxidative stress in HK-2 cells when exposed to iohexol

To further research the role of HDAC9 in the susceptibility of high glucose to contrast media-induced injury, contained HDAC9 siRNA or control siRNA (NC) were used in this study. After screening three siRNAs, we selected the third siRNA (HDAC9-siRNA 3) with the highest knockdown efficiency for the following experiment (Figure 5A). Compared with the HG+CM group, the Western blot showed that the expression of the pro-apoptotic protein cleaved-caspase 3 was significantly reduced in renal tubular epithelial cells after knockdown of HDAC9 (Figures 5B and 5C, $^{\#\#}p<0.01$ vs HG+CM), the results may suggest that exposure of HK-2 cells to high glucose by knocking down HDAC9 can attenuate further damage to apoptosis by iohexol. Also, to verify whether knockdown of HDAC9 could attenuate the level of oxidative stress of HK-2 in cells co-cultured with high glucose and iohexol, we used the reactive oxygen fluorescent probe (H2-DCFDA) to observe the effect of HK-2 transfection with siRNA-HDAC9 followed by exposure to high glucose and iohexol (HG+CM+si-HDAC9) on cellular oxidative stress. The results showed that HDAC9 knockdown significantly reduced ROS production in HK-2 compared to HG+CM or HG+CM+Scramble groups (Figure 5D). In conclusion, the above experiment indicated that knockdown of HDAC9

significantly attenuated the level of apoptosis and oxidative stress induced by HK-2 under high glucose and iohexol co-incubation.

3.6 HDAC9 promotes TXNIP/Trx1/P-ASK1/p38MAPK pathway activation in HK-2 cells induced by the combination of HG and iohexol

It has been shown that glucose can regulate TXNIP expression in normal human mesangial cells by epigenetic modifications and histone deacetylase inhibitors are involved in glucose-regulated TXNIP expression [32]. However, there would be no further increase in TXNIP expression when the use of HDAC inhibitors at higher glucose concentrations(30). Hence, to investigate whether HDAC9 can regulate TXNIP production under high glucose stimulation, we knocked down HDAC9 in HK-2 and exposed to high glucose and iohexol, then detected its expression by Western blotting. Compared with HG + CM group, the expression of TXNIP protein in HG+CM+si-HDAC9 group was significantly decreased (Figures 6A and 6B, $^{\#}p<0.05$ vs HG+CM). However, when overexpress TXNIP, cells showed lower Trx1 activity, as shown in Figure 6A in HG+CM group (Figure 6C, $^{\#}p<0.05$ vs HG+CM). Furthermore, it also led to the activation of P-ASK1 (Figures 6A and 6D, $^{\#}p<0.05$ vs HG+CM); at the same time to activate its downstream substrates p38MAPK (Figures 6A and 6E, $^{\#}p<0.05$ vs HG+CM) and finally initiated the apoptotic pathway (Figures 6A and 6F, $^{\#}p<0.05$ vs HG+CM). After knockdown HDAC9, the expression of TXNIP decreased

in HK-2 cells when exposed to high glucose and iohexol, while the expression of TRX1 increased and the activation of P-ASK1 decreased, the activation of p38MAPK was also decreased, and the expression of pro-apoptotic protein was decreased obviously.

3.7 HDACI (HDAC9 inhibitor) improves renal function and attenuates oxidative stress, apoptosis in diabetic-CIAKI mice

HDACI injection group significantly increased the GSH-PX (Figure 7A) and SOD (Figure 7B) activities and decreased MDA (Figure 7C) levels ($^{\#}p<0.05$ vs DM-CIAKI, $^{\#\#}p<0.01$ vs DM-CIAKI). In addition, dihydroethidium (DHE) staining was used to determine the effect of HDACI on ROS levels in the kidney. Compared to the diabetic-CIAKI, a low level of red fluorescence intensity was shown in the kidney after HDACI injection (Figure 7D). The result suggests that HDAC9 inhibitor significantly inhibited the accumulation of ROS in the kidneys of diabetic-CIAKI mice, thereby reducing the level of renal oxidative stress. Furthermore, apoptosis was assessed with TUNEL staining method in kidney sections. Compared to CIAKI mice, Diabetic-CIAKI mice exhibited markedly increased numbers of TUNEL-positive tubular cells (Figure 7E). Pretreatment with HDACI in Diabetic-CIAKI mice decreased apoptotic cell numbers (Figure 7E). Meanwhile, HDACI pretreatment also reduced histological injury to the renal tubules of Diabetic-CIAKI mice, as illustrated by H&E staining and semiquantitative scoring of the pathological lesion. (Figure 7F). In

addition, HDACI pretreatment in Diabetic-CIAKI mice was verified by less increases in SCr and BUN in mice than Diabetic-CIAKI group (Figure 7H). Overall, these findings suggest that HDACI may protect against kidney injury by scavenging ROS and inhibiting apoptosis to improve renal function.

3.8 HDACI inhibitor weakens TXNIP/Trx1/P-ASK/p38 pathway activation in Diabetes-CIAKI mice

Experiments in vitro have demonstrated that HK-2 cells exposed to high glucose expressing HDAC9 may be involved in the activation of the TXNIP/Trx1/P-ASK1/p38MAPK pathway. To investigate whether this pathway can be inhibited by HDACI in vivo, HDAC9 inhibitors were injected intraperitoneally in diabetic-CIAKI mice, and western blotting results shown to be consistent with the results of in vitro experiments. That is, HDAC9 inhibitor injection significantly decreased TXNIP protein expression and increased Trx1 activity compared with the diabetic-CIAKI mice group, thereby decreasing P-ASK1 enzyme activity resulting in reduced activation of the substrate p38MAPK and ultimately a significant decrease in pro-apoptotic protein expression levels (Figure 8A).

4.Discussion

There is mounting evidence that diabetes mellitus (DM) is a chronic metabolic disorder and considered as an important risk factor for CI-AKI. Preprocedural hyperglycemia is associated with CI-AKI and could prolong

hospital stays. With the frequent application of contrast agents, contrast nephropathy is the third leading cause of hospital-acquired acute kidney injury(2, 3). Many mechanisms relate to renal toxicity of contrast media, including renal medullary hypoxia, direct toxicity of contrast agents, oxidative stress, apoptosis, and immunity/inflammation(9, 36, 37). Chronic hyperglycemia led to upregulation of reactive oxygen species (ROS) (38, 39) and inflammatory cytokines, in turn, contributing to the development of CIAK. Although there exist various factors inducing diabetes sensitive to CIAKI. the exact molecular mechanisms need to be further explored. The major findings in our study, are as follows: (I) HDAC9 knockdown alleviates HK-2 cells (in vitro) injury co-treated with both high glucose and iohexol, (II）HDAC9 inhibitor protects the diabetic mice against contrast-induced acute kidney injury, (III) HDAC9 knockdown effectively attenuates oxidative stress, apoptosis caused by co-cultured with high glucose and iohexol through decreasing TXNIP expression, and inhibiting ASK1/p38MAPK pathway. In the present study, we researched the effects of HDAC9 on HK-2 and also the diabetic mice predisposed to CIAKI, our results revealed that exposure to high glucose then administration of iohexol created a vicious state, leading to more injuries in cells. When HDAC9 was inhibited or knockdown in HK-2 co-treated with solutions, low levels of apoptosis and oxidative stress occurs. Meanwhile, the Diabetic-CIAKI mice showed heavier renal dysfunction

than CIAKI mice alone, as confirmed by increased level of biochemical marker (SCr and BUN), aggravated histopathological injury and increasing ROS level in kidney. However, these phenomena were mitigated by the treatment with HDAC9 inhibitor in the Diabetic-CIAKI mice.

Wang et al [48] showed that HDAC2/4/5 expression was upregulated in the kidneys of streptozotocin-induced diabetic rats, diabetic db/db mice, and in kidney biopsies from diabetic patients(40). Another experiment showed that upregulation of HDAC9 was found to mediate podocyte injury and promote glomerulosclerosis in diabetic nephropathy mice (31), while analysis of GEO data also showed that HDAC9 expression was upregulated in renal tissue of diabetic nephropathy patients (41). Experimental rat and mice models of cerebral ischemia reperfusion (I/R) injury demonstrated that HDAC9 contributes to brain microvessel endothelial cell dysfunction (42, 43). A case-control study also suggested that people who has a history of diabetes with the combination of the HDAC3 and HDAC9 genes, would increase the risk of ischemic stroke (44). In our study, we observed the increased level of HDAC9 in both the diabetic mice of renal tubulars and high-glucose conditioned HK-2 cells. Based on the above basic experimental researches, they may provide a new clue to explain why diabetes is a high risk for CIAKI. The specific mechanisms underlying diabetes being a risk factor for CI-AKI are not fully illustrated, although the coenzyme Q-10 (CoQ10) (45) and physical

training (PT) have been confirmed to have renoprotective effects on CI-AKI in diabetic rats by reducing oxidative stress and improving renal function. In our research, ROS level was remarkably increased in HK-2 cells co-cultured with high glucose and iohexol (HG+CM) and also in the Diabetic-CIAKI mice kidneys, which can mediate oxidative stress and cause cytotoxic damage. Furthermore, antioxidant enzyme activities including SOD, GSH-Px were prominent decreased and treatment with HDCAI alleviated these tendencies compared with Diabetic-CIAKI mice. The results were consistent with HK-2 cells. Interestingly, we found gene silencing of HDAC9 significantly reduced the HK-2 cells (HG+CM) apoptosis via decreasing the cell apoptosis. Meanwhile, HDAC9 knockdown in HK-2 cells also attenuated the oxidative stress by reducing the ROS. The same findings were observed in Diabetic-CIAKI treatment with HDAC inhibitor.

Previous evidences have been proved p38MAPK activation is related to renal injury, such as iohexol-induced(29, 46, 47) or diabetes-induced(48-51) apoptotic cell death. MAPK kinase (MAPK3k) acts as an upstream regulator of MAPKs. Apoptosis signal-regulating kinase 1 (ASK1) is a widely expressed redox-sensitive serine threonine kinase. Studies have shown that activation of ASK1 (as a member of the MAP3K family) can target its substrate p38 MAPK, which is involved in the development of renal fibrosis (52), glomerular injury (53), tubular epithelial cell apoptosis,

and inflammation(29). Recently, a new study found a significant increase in the expression of ASK1 and P38 in patients' venous tissues in maintenance hemodialysis patients, and p38MAPK signaling pathway may be involved in failure of autogenous arteriovenous fistula caused by stenosis (54). In renal biopsies with diabetic kidney disease (DKD) patients, activation ASK1 pathway in the glomerular and tubular has been confirmed by JT et al, further analysis with GS-444217 (a selective small-molecule inhibitor of ASK1) treatment in several rodent models, showed reduced inflammation and fibrosis in renal tissues(55). In our present data, we observed the higher level of ASK1/p38MAPK in HK-2 cells (HG+CM) and the Diabetic-CIAKI mice than CM in HK-2 cells group and the CIAKI mice group. However, HDAC9 knockdown reversed the increased level of ASK1/p38MAPK both in HG+CM groups and HDACI treatment in the Diabetic-CIAK mice. Therefore, we can conclude the ASK1/p38 MAPK pathway may be involved in the pathogenesis of contrast nephropathy susceptibility in diabetes mellitus. Additionally, decreased Trx1 and increased ASK1 phosphorylation have been verified in CIAKI rat model. It is known that reduced Trx1 acts a negative regulator of ASK1. We also found the downregulation of Trx1 in HG+CM cells and the Diabetic-CIAKI mice, yet HDAC9 gene silencing could increase the Trx1 in HG+CM cells. The same phenomenon was observed with HDACI treatment in Diabetic-CIAKI mice. As discussed above, it is further

confirmed that HDAC9 may contribute to the molecular mechanism of diabetic susceptibility to CIAKI by regulating Trx1/ASK1/p38MAPK pathway.

Thioredoxin-interacting protein (TXNIP), a pro-oxidant protein has been identified in various diseases, involving in diabetes mellitus (23, 56-59), renal disease(21, 23, 57, 60-62), and cardiovascular disease (63-66). TXNIP expression is regulated by different cellular stress factors, these contain hypoxia, ROS, nitric oxide, UV light, high glucose (HG) and basic cellular factor(26, 67). TXNIP was associated with vascular complications of diabetes and inhibits the antioxidant activity of thioredoxin (TRX), which finally exacerbating oxidative stress. Numerous data demonstrate the destructive role of TXNIP in diabetic nephropathy, but little research has been done on the involvement of TXNIP in CIAKI disease. In our data, exposure to contrast media increased protein TXNIP expression in HK-2 cells; similarly, experiments in mice confirmed that TXNIP expression levels were higher in CIAKI mice compared to control mice. A study showed glomerular mesangial cell exposure to hyperglycemia can stimulate TXNIP expression by epigenetic regulation, which means increased level of TXNIP is associated with activation histone marks H3K9ac, H3K4me3, and H3K4me1 at the gene promoter region (30). In our study, we found knockdown of HDAC9 reduced high glucose-induced TXNIP expression, thereby attenuating oxidative stress and apoptosis

production in renal tubular epithelial cells. The Diabetic-CIAKI mice treatment with HDCAI also showed the nephroprotective effects. We have reasons to believe HDAC9 may engage the regulation of TXNIP in high glucose. In summary, the apoptosis and oxidative stress effect via the HDAC9-TXNIP-Trx1-P-ASK1-p38MAPK axis may be an important mechanism of diabetes susceptibility to contrast nephropathy.

5. Conclusion

In summary, this study found HDAC9 was increased in both diabetic kidney tissues and HG-induced HK-2 cells; After knocking down or suppressing HDAC9, HK-2 cells exposure to HG could reduce the apoptosis induced by iohexol and finally increase the activity of HK-2 cells. At the same time, HDAC9 inhibitors can protect diabetic mice from CIAKI by alleviating apoptosis and ROS production in kidney tissues, which may be regulated by TXNIP-Trx1-P-ASK1-p38MAPK signaling pathways. Our study provides novel insight into the mechanism underlying HDAC9 in the susceptibility of diabetes to CIAKI.

Abbreviations

CIAKI Contrast-induced acute kidney injury

DM Diabetes

HDAC9 Histone deacetylase 9

ROS Reactive oxygen species

HG high glucose

CM Contrast media

HK-2 human renal tubular epithelial cells

C-CAS3 Cleaved-caspase 3

TXNIP Thioredoxin-interacting protein

Trx1 Thioredoxin 1

P-ASK1 Phosphorylated-Apoptosis signal regulating kinase 1

P-p38MAPK Phosphorylated-p38-mitogen-activated protein kinase

HE Hematoxylin-eosin

Tunel Terminal-deoxynucleoitidyl transferase mediated nick end labelin

CCK-8 Cell Counting Kit-8

ANOVA Analysis of variance

Declarations

Author Contributions

LH and HL designed the experiments; LH analyzed the data and drafted the article; LH, DW, LZ conducted the experiment; XL, Liu H, SL gave conceptual advice on data interpretation and experimental technology. HL revised the paper critically for important intellectual content. All authors read and approved the final manuscript.

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Availability of data and materials

The authors agree the data supporting the findings of this study are available upon requests.

Ethics approval and consent to participate

All animal experimental procedures followed the guideline of the Ethical Committee of the Second Xiangya Hospital, Central South University.

Consent for publication

Not applicable

Competing interest

All authors declare no conflict of interest

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Figure legends

Figure 1 Sensitivity of STZ-induced diabetic mice to contrast-induced acute kidney injury.

(A-C) C57BL/6J mice were injected with STZ to induce diabetes (DM) or injected with vehicle (ND: nondiabetic). Then mice received tail vein injection of iohexol and execution after 24 hours. Blood samples were collected to measureSCr (A) and BUN (B). Representative histology (C) was examined by collecting kidney tissue for hematoxylin and eosin staining（D）Renal tubular pathological score. BUN, blood urea nitrogen; DM, diabetes mellitus; STZ, streptozotocin. *$p<0.05$ vs Con，**$p<0.01$ vs Con，$^{\#}p<0.05$ vs DM-CIAKI，$^{\#\#}p<0.01$ vs DM-CIAKI，$^{\&}p<0.05$ vs CIAKI

Figure 2 Contrast media (iohexol) accelerates HG-induced HK-2 cell apoptosis

The HK-2 cell morphology and hochest staining under two conditions: contrast media (CM) and high glucose+contrast media (HG+CM). (B) The cell viability of HK-2 cells detected by CCK-8 assay. (C) The apoptosis protein of cleaved caspase-3 by western blotting. (D) Western blotting semi-quantitative analysis of cleaved caspase-3.

Con*p<0.05 vs control，**p<0.01 vs control，&p <0.05 vs CM，#p<0.05 vs CM+HG，##p<0.01 vs CM+HG

Figure 3 HK-2 exposed to high-glucose as well as tubular epithelial cells in diabetes mice increase HDAC9 expression

Cultured HK-2 was treated with high glucose and (or) iohexol analyzed by Western Blotting with anti-HDAC9 (A and C) and Tubulin (A and C) antibodies. Representative western blot image and densitometric quantification was shown (B). The four groups mice analyzed by western blotting with anti-HDAC9 (D) and Tubulin (D) antibodies. *P < 0.05 vs 5.5 mM D-glucose control, n=3.

Figure 4 HDACI attenuates oxidative stress and apoptosis in HK-2 cultured with high glucose and iohexol

(A) Detection of HK-2 cell proliferation in five groups by CCC-8 kit and Western blotting experiments with cleaved-caspase-3 antibody (B). (C) Cell morphology under light microscopy and ROS (reactive oxygen species) under fluorescence microscopy: including HK-2 contrast media group (CM), high glucose + contrast media group (HG + CM), high glucose+ HDAC9 inhibitor + contrast media group (HG + CM+HDACI）. *p<0.05 vs Con，**p<0.01 vs Con，#p<0.05 vs HG+CM，##p<0.01 vs HG+CM，&p<0.05 vs HG+CM+HDACI.

Figure 5 Knockdown of HDAC9 reduced ROS, apoptosis in HK-2 co-treated with high glucose and iohexol treatment.

(A) HDAC9 was silenced using HDAC9-siRNA. The result of western blot analyses confirmed that HDAC9 protein expression levels were knocked down by HDAC9-siRNA. The effects of HDAC9 knockdown on the levels of cell apoptosis (B) and ROS production under immunofluorescence microscopy (C) in HK-2 with high glucose and iohexol treatment.

Figure 6 Effect of HDAC9 knockdown on TXNIP/Trx1/P-ASK1/p38MAPK

pathway activation in HK-2 under high glucose and iohexol co-treatment

(A) Western blotting (B) TXNIP (C) Trx-1 (D) P-ASK1/ASK1 (E) P-p38MAPK/p38MAPK (F) C-CAS3/Gapdh semi-quantitatively analyzed in representative blot images. *p<0.05 vs Con，**p<0.01 vs Con，#p<0.05 vs HG+CM，##p<0.01 vs HG+CM，&&p<0.01 vs CM, ᵃᵃp<0.01 vs HG+CM+si-HDAC9

Figure 7 HDACI (HDAC9 inhibitor) improves renal function and attenuates oxidative stress, apoptosis in diabetic-CIAKI mice

(A) Injected with iohexol via tail in diabetic mice significantly attenuated GSH-Px and, (B) SOD and (C) increased MDA level. (D) Representative DHE staining and (E) TUNEL staining in kidney. (F) Representative images of H&E staining showing that HDACI pretreatment alleviated renal pathological injury at 24 h after Diabetic-CIAKI. (G) Pathological damage scores of H&E-stained sections showing the beneficial effects of HDACI in mitigating in Diabetic-CIAKI mice. (H) Biochemical indicators related to renal function SCr and BUN. The values are means ± SD (n = 6). *p<0.05 vs Con，**p<0.01 vs Con，$^{\#}$p<0.05 vs DM-CIAKI，$^{\#\#}$p<0.01 vs DM-CIAKI

Figure 8 HDAC inhibitor weakens pathway TXNIP/Trx1/ASK1/p38MAPK pathway activation in Diabetes-CIAKI mice

(A)Western blotting (B) TXNIP (C) Trx-1 (D) P-ASK1/ASK1 (E) P-p38MAPK/p38MAPK semi-quantitative analysis in representative blot images. *p<0.05 vs Con，**p<0.01 vs Con，$^{\#}$p<0.05 vs DM-CIAKI，$^{\#\#}$p<0.01 vs DM-CIAKI，$^{\alpha\alpha}$p<0.01 vs DM+hdi+CIAKI

Figures

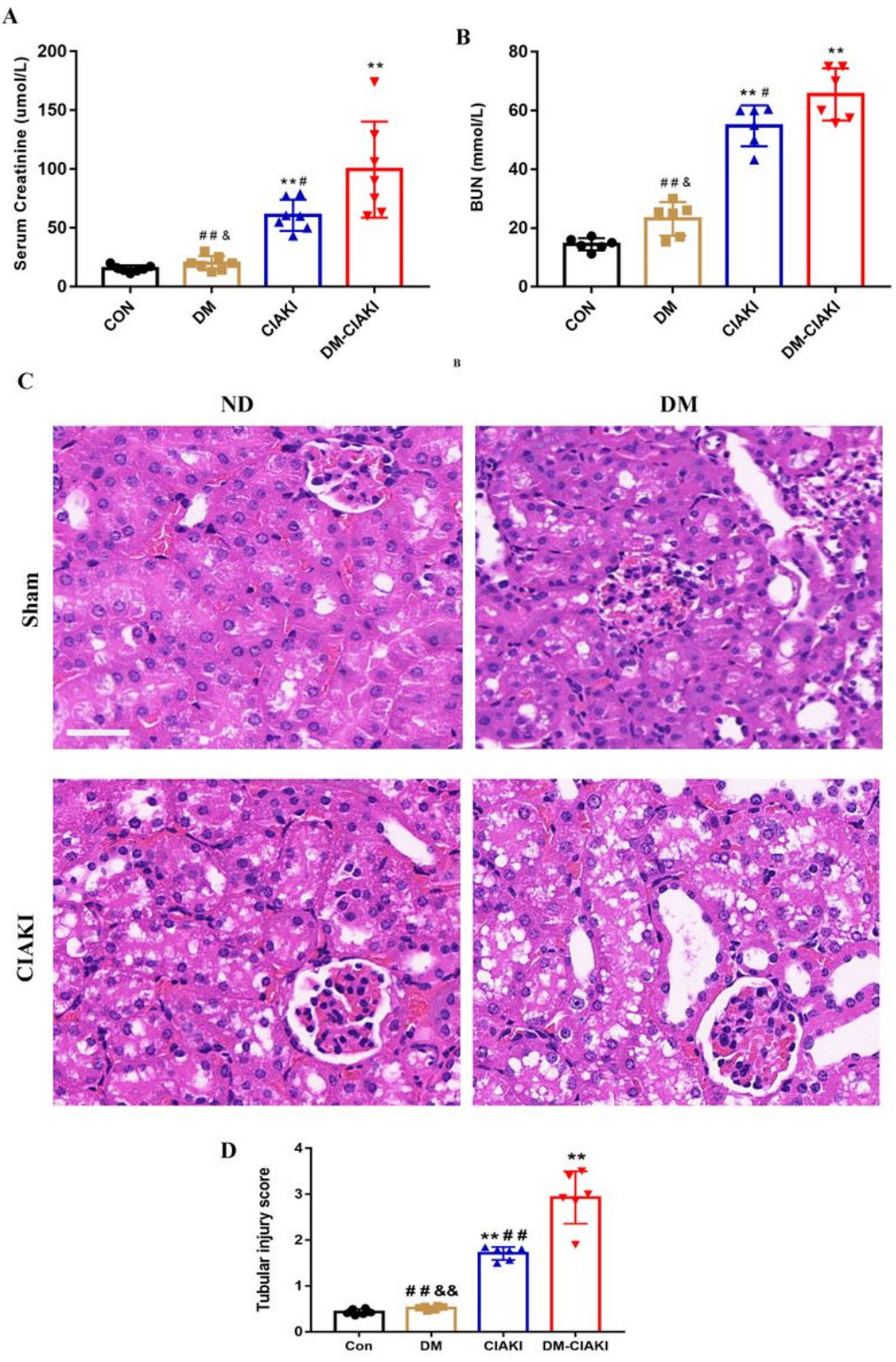

Figure 1

Sensitivity of STZ-induced diabetic mice to contrast-induced acute kidney injury. (A-C) C57BL/6J mice were injected with STZ to induce diabetes (DM) or injected with vehicle (ND: nondiabetic). Then mice received tail vein injection of iohexol and execution after 24 hours. Blood samples were collected to

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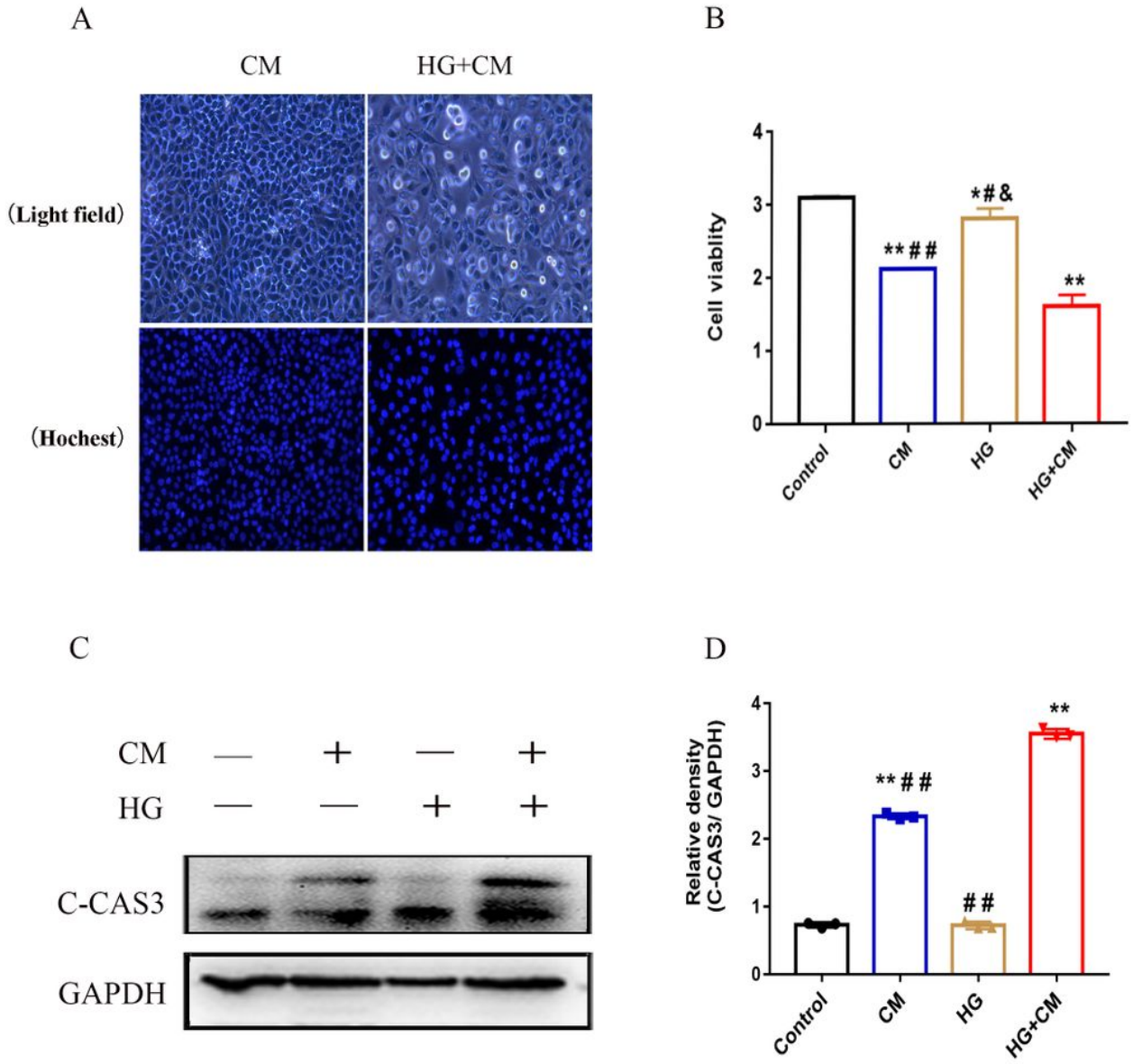

Figure 2

Contrast media (iohexol) accelerates HG-induced HK-2 cell apoptosis The HK-2 cell morphology and hochest staining under two conditions: contrast media (CM) and high glucose+contrast media (HG+CM). (B) The cell viability of HK-2 cells detected by CCK-8 assay. (C) The apoptosis protein of cleaved caspase-3 by western blotting. (D) Western blotting semi-quantitative analysis of cleaved caspase-3. Con*p<0.05 vs control（**p<0.01 vs control（&p <0.05 vs CM（# p<0.05 vs CM+HG（ # # p<0.01 vs CM+HG

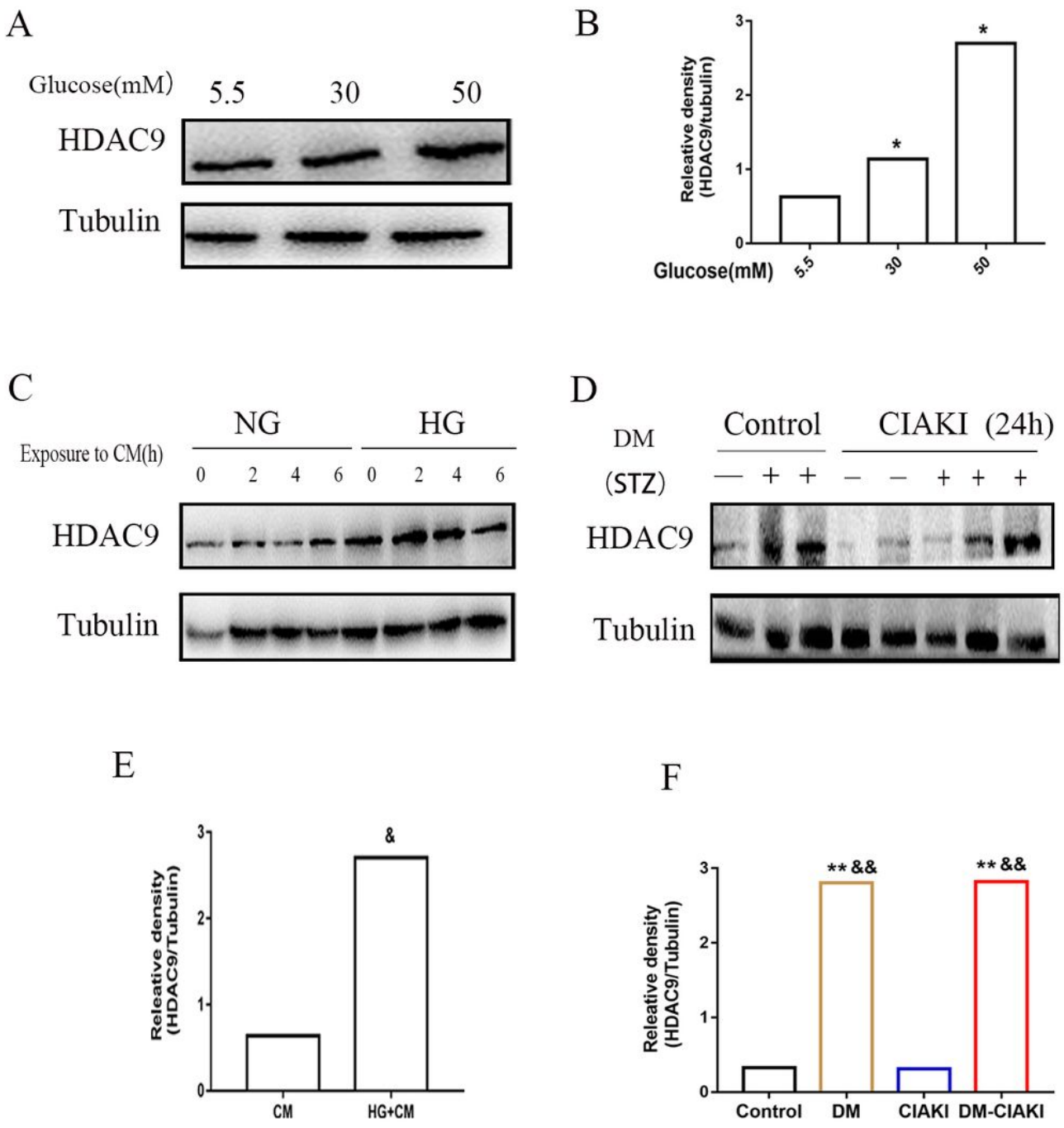

Figure 3

HK-2 exposed to high-glucose as well astubular epithelial cells in diabetes mice increase HDAC9 expression Cultured HK-2 was treated with high glucose and (or) iohexol analyzed by Western Blotting with anti-HDAC9 (A and C) and Tubulin (A and C) antibodies. Representative western blot image and densitometric quantification was shown (B). The four groups mice analyzed by western blotting with anti-HDAC9 (D) and Tubulin (D) antibodies. *P < 0.05 vs 5.5 mM D-glucose control, n=3.

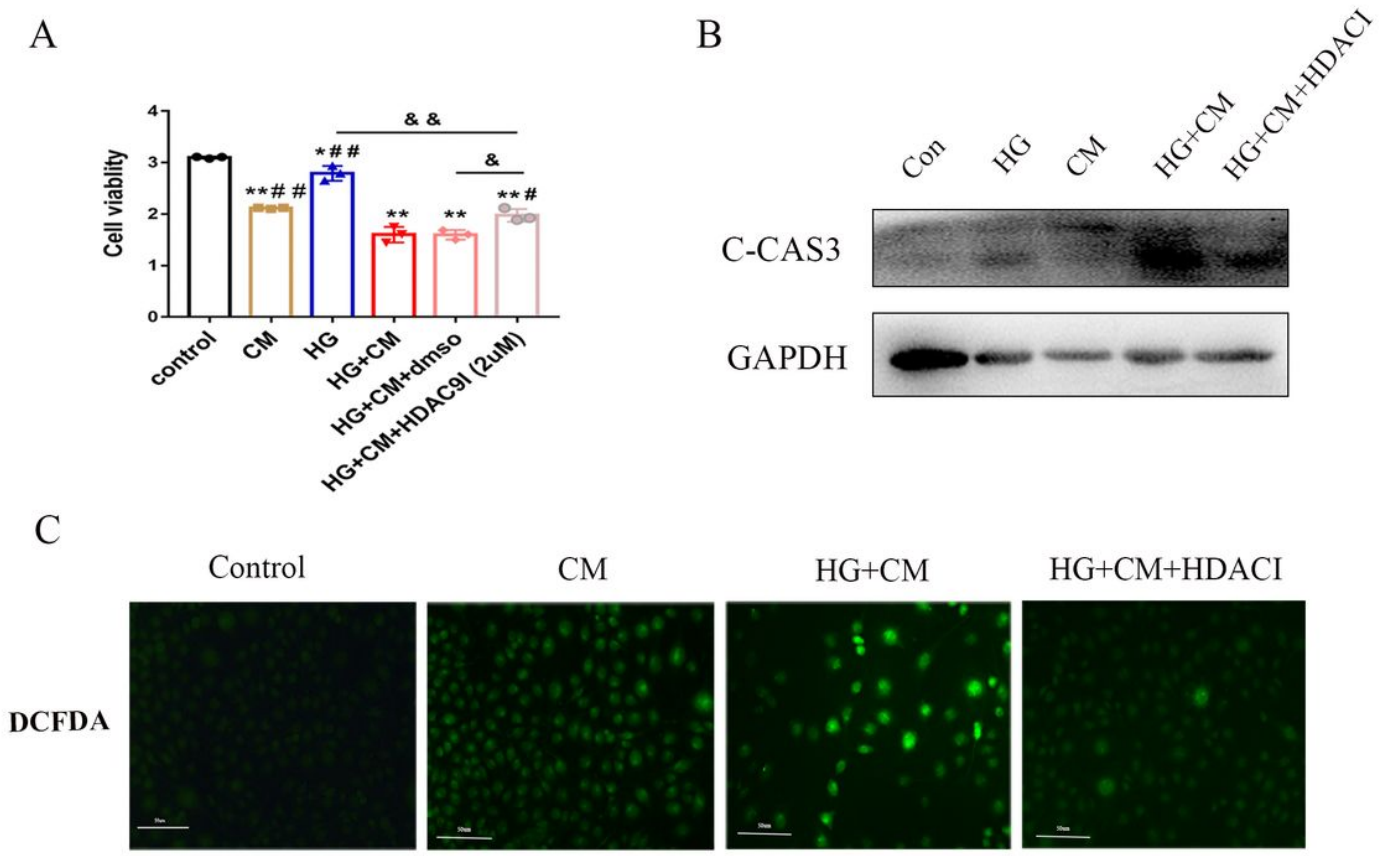

Figure 4

HDACI attenuates oxidative stress and apoptosis in HK-2 cultured with high glucose and iohexol (A) Detection of HK-2 cell proliferation in five groups by CCC-8 kit and Western blotting experiments with cleaved-caspase-3 antibody (B). (C) Cell morphology under light microscopy and ROS (reactive oxygen species) under fluorescence microscopy: including HK-2 contrast media group (CM), high glucose + contrast media group (HG + CM), high glucose+ HDAC9 inhibitor + contrast media group (HG + CM+HDACI. *p<0.05 vs Con□**p<0.01 vs Con□# p<0.05 vs HG+CM□# # p<0.01 vs HG+CM□&p<0.05 vs HG+CM+HDACI.

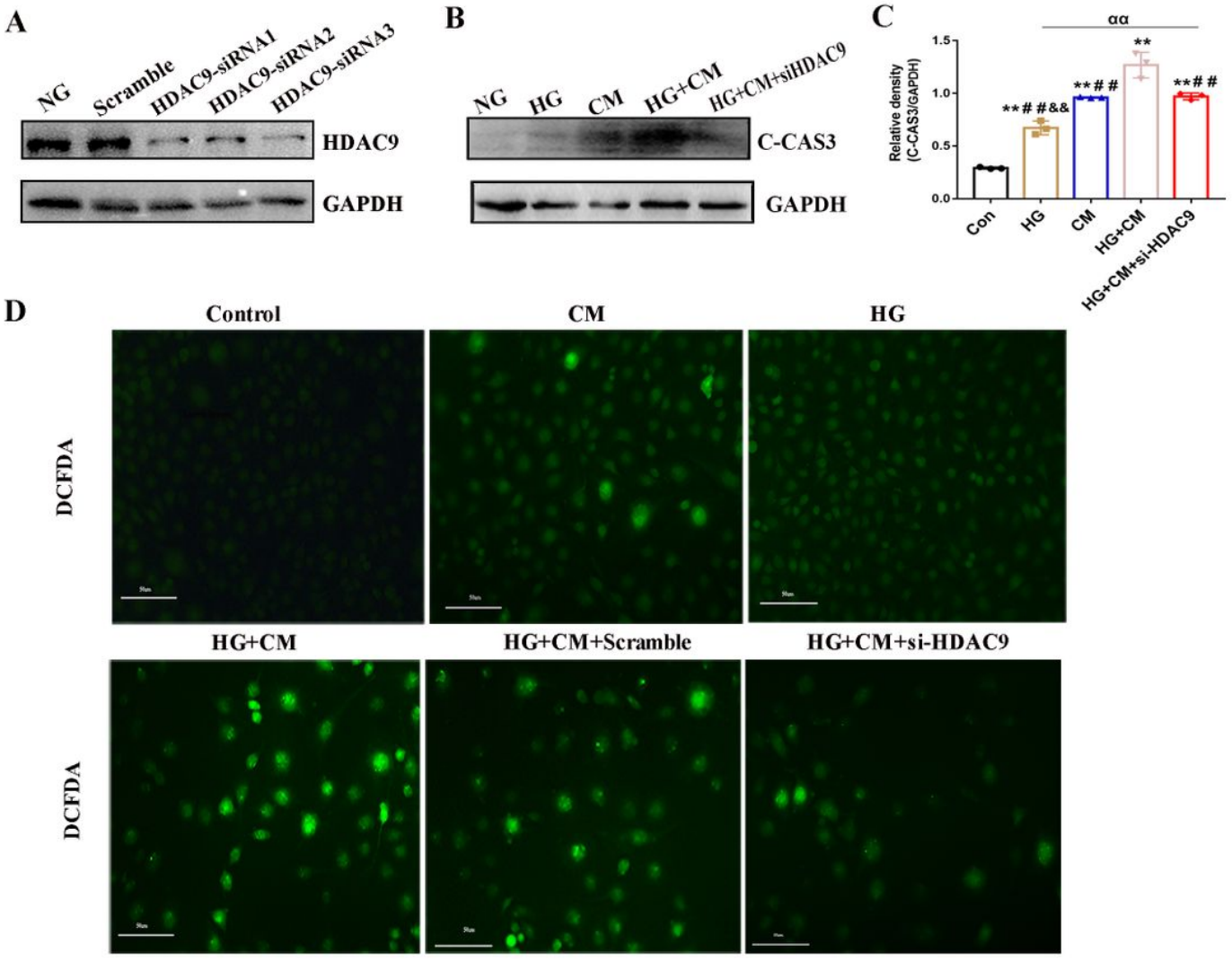

Figure 5

Knockdown of HDAC9 reduced ROS, apoptosis in HK-2 co-treated with high glucose and iohexol treatment. (A)HDAC9 was silenced using HDAC9-siRNA. The result of western blot analyses confirmed that HDAC9 protein expression levels were knocked down by HDAC9- siRNA. The effects of HDAC9 knockdown on the levels of cell apoptosis (B) and ROS production under immunofluorescence microscopy (C) in HK-2 with high glucose and iohexol treatment.

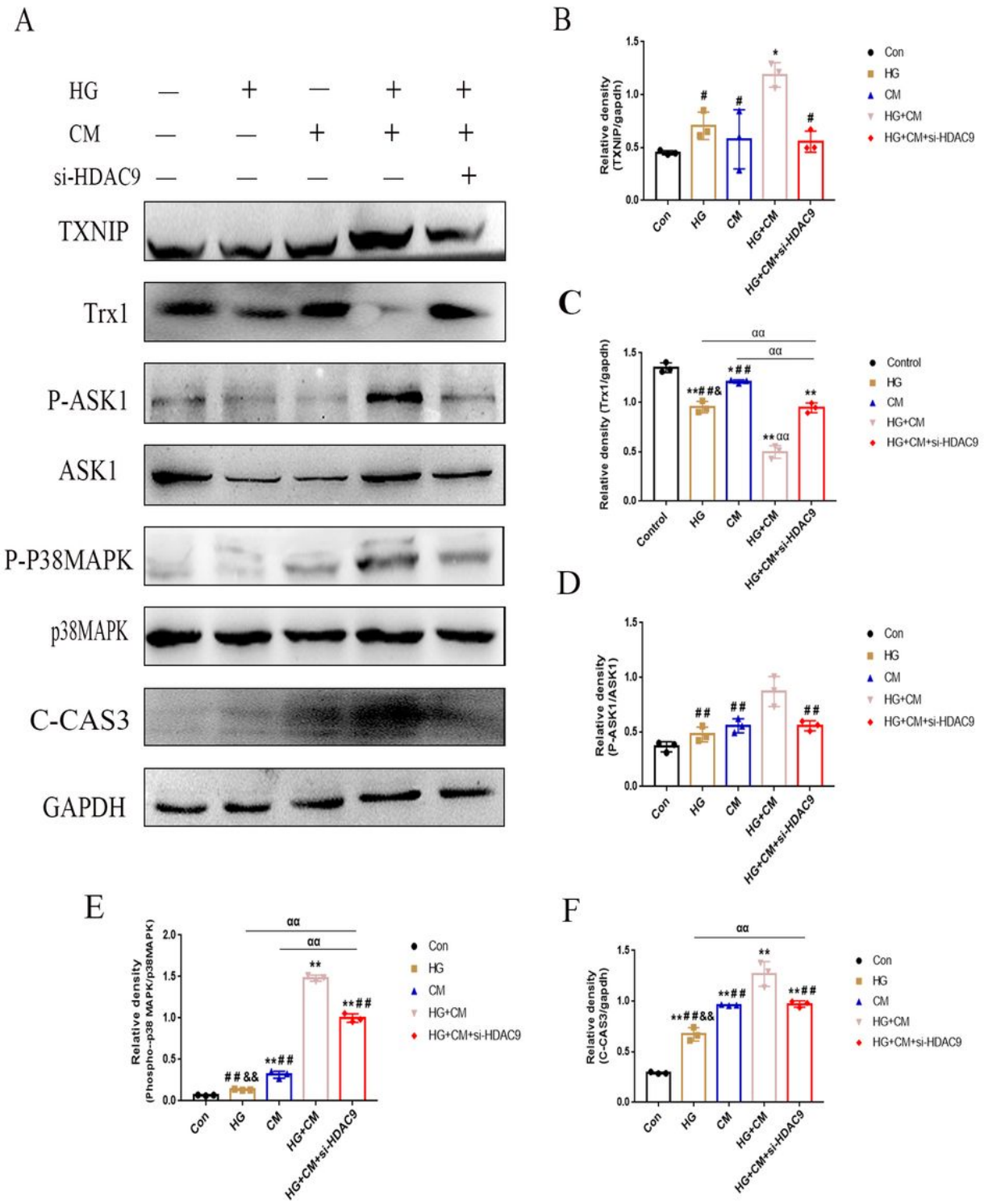

Figure 6

Effect of HDAC9 knockdown on TXNIP/Trx1/P-ASK1/p38MAPK pathway activation in HK-2 under high glucose and iohexol co-treatment (A) Western blotting (B) TXNIP (C) Trx-1 (D) P-ASK1/ASK1 (E) P-p38MAPK/p38MAPK (F) C-CAS3/Gapdh semi-quantitatively analyzed in representative blot images. *p<0.05 vs Con□**p<0.01 vs Con,# p<0.05 vs HG+CM, # # p<0.01 vs HG+CM,&&p<0.01 vs CM, αα p<0.01 vs HG+CM+si-HDAC9

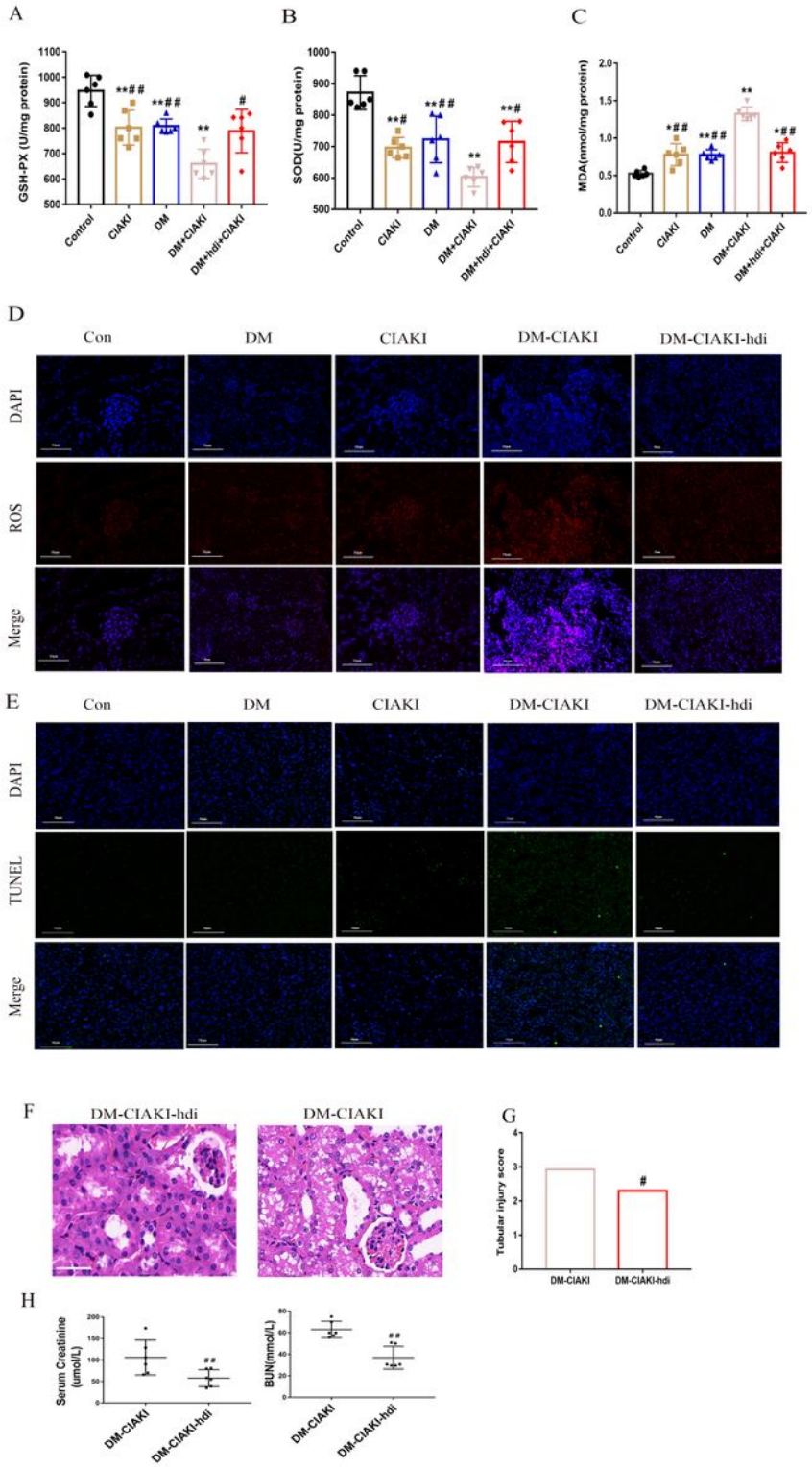

Figure 7

HDACI (HDAC9 inhibitor) improves renal function and attenuates oxidative stress, apoptosis in diabetic-CIAKI mice (A) Injected with iohexol via tail in diabetic mice significantly attenuated GSH-Px and, (B) SOD and (C) increased MDA level. (D) Representative DHE staining and (E) TUNEL staining in kidney. (F) Representative images of H&E staining showing that HDACI pretreatment alleviated renal pathological injury at 24 h after Diabetic-CIAKI. (G) Pathological damage scores of H&E-stained sections showing the

beneficial effects of HDACI in mitigating in Diabetic-CIAKI mice. (H) Biochemical indicators related to renal function SCr and BUN. The values are means ± SD (n = 6). *p<0.05 vs Con,**p<0.01 vs Con,# p<0.05 vs DM-CIAKI,# # p<0.01 vs DM-CIAKI

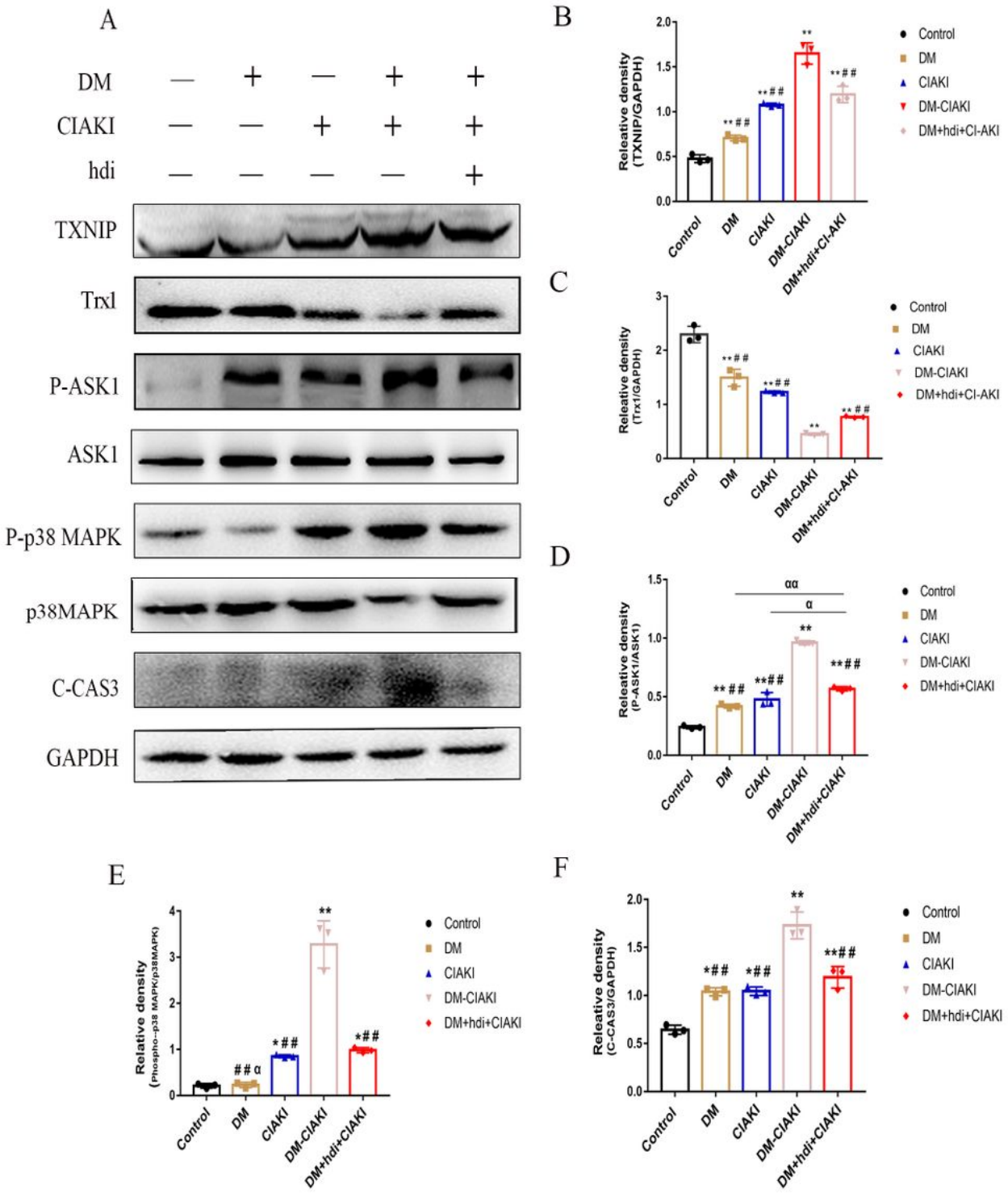

Figure 8

HDAC inhibitor weakens pathway TXNIP/Trx1/ASK1/p38MAPK pathway activation in Diabetes-CIAKI mice (A)Western blotting (B) TXNIP (C) Trx-1 (D) P-ASK1/ASK1 (E) P-p38MAPK/p38MAPK semi-quantitative analysis in representative blot images. *p<0.05 vs Con,**p<0.01 vs Con,# p<0.05 vs DM-CIAKI# # p<0.01 vs DM-CIAKI, αα p<0.01 vs DM+hdi+CIAKI