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Angiotensin II type 2 receptor and angiotensin-converting enzyme 2 mediate ischemic renal injury in diabetic and non-diabetic rats

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ABSTRACT

Aim: Depressor arm of the renin-angiotensin system (RAS) exerts reno-protective effects in chronic kidney diseases like diabetic nephropathy. However, same is still elusive under AKI and hyperglycaemia comorbidity. Hence, the present study delineates the role of angiotensin-II type 2 receptor (AT2R) and angiotensin-converting enzyme 2 (ACE2) in AKI under normal and hyperglycaemia condition.

Methods: Non-diabetic (ND) and Streptozotocin-induced diabetes mellitus (DM) rats were subjected to ischemic renal injury (IRI). Rats underwent IRI were treated with an AT2R agonist, C21 (0.3 mg/kg/day, *i.p.*) or ACE2 activator, Dize, (5 mg/kg/day, *p.o.*) either alone or as combination therapy. Renal histopathology and immunohistochemistry, proximal tubular fraction isolation, ELISA, immunoblotting and qRT-PCR were performed for subsequent analysis.

Key findings: Rats subjected to IRI displayed an increase in plasma ACE, AT1R, AT2R, Ang II, and reduction in ACE2, Ang-(1–7) expressions, with augmented renal inflammation and apoptosis. These changes were more prominent in diabetic rats with IRI. Co-administration of C21 and Dize augmented ACE2, Ang-(1–7), AT2R and MasR expressions, and attenuated tubular injury in both DM and ND rats.

Conclusion: We demonstrated that pharmacological activation of AT2R and ACE2 protects DM and ND rats from IRI by preventing oxidative stress, inflammation and apoptosis-mediated tubular damage.

1. Introduction

Clinically, acute kidney injury (AKI) is considered a catastrophic condition with incidences allied with high morbidity and mortality [1,2]. One of the major risk factors for AKI is diabetes mellitus (DM) [3,4]. As compared to non-diabetics, diabetic patients remain on the higher risk of AKI [5]. Adverse renal outcomes in DM and AKI individually are attributed mainly to the renin-angiotensin system (RAS) driven activation of mitogen-active protein kinase (MAPK)-mediated apoptosis, NF-κB mediated inflammation, and redox imbalance promoting oxidative stress [6–8]. Therefore, RAS inhibition via angiotensin II receptor blockers (ARB) or angiotensin-converting enzyme inhibitors (ACEi) was thought to prevent AKI. Surprisingly, it was observed that when ARB and ACEi were used to treat AKI patients, they increased the severity of AKI [8]. Hence, it highlights the need for new therapeutic approaches for the treatment of AKI.

The RAS is comprised of two counter regulatory arms, the pressor arm [angiotensin II (Ang-II)/angiotensin-converting enzyme (ACE)/Ang II type 1 receptor (AT1R)] and the depressor arm (Ang-(1–7)/ACE2/MasR), which induces vasoconstriction and vasodilation, respectively, thereby helping in maintaining blood pressure and other homeostasis [8]. Interestingly, the pressor arm of the RAS has been explored in depth concerning the pathophysiology and pharmacotherapy of the renal complications including AKI. However, no study has demonstrated the role of the depressor arm of RAS in the pathogenesis of AKI-DM co-morbidity till date; hence the present study focuses the same.

The depressor arm of RAS sets a compensatory mechanism by sensing the elevated cellular stress and pathological signalling, thus benefits the renal system [9,10]. Tourniquet-induced remote kidney injury mice demonstrated deregulation of ACE and ACE2 activity, which cause kidney damage. Interestingly, restoring the ACE/ACE2 balance by up-regulation of ACE2 prevented renal damage [11]. Lipopolysaccharide-induced endotoxemia significantly decreased mRNA levels of ACE2 and

Abbreviations: ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; AKI, acute kidney injury; Ang II, angiotensin II; Ang-(1–7), angiotensin-(1–7); AT2R, angiotensin II type 2 receptor; DM, diabetes mellitus; IRI, ischemic renal injury; ND, non-diabetic; RAS, renin-angiotensin system

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elevated Ang II, and inducible nitric oxide synthase levels in kidney, whereas restoration of ACE2 levels maintained endothelial porosity, glomerular filtration rate, and proximal tubular function, eventually protects the kidney against AKI [12]. Ruiz-Ortega et al. demonstrated overexpression of AT2R in renal tubular cells of Balb/c subjected to folic acid-induced AKI and proposed the potential role of AT2R in protecting the kidney damage [13]. Subsequently, overexpression of AT2R and activation of ACE2 activity significantly improved the renal function of mice in these models [11–13].

Therefore, in the present study, we hypothesised that the pharmacological activation of AT2R and ACE2 might protect rats from ischemic renal injury (IRI). Since hyperglycaemia remains a major risk factor for ischemic AKI, we also evaluated the protective effect of AT2R and ACE2 activation in diabetic rats with IRI.

2. Materials and methods

2.1. Materials

Streptozotocin and diminazene aceturate (Dize) were obtained from Sigma (St. Louis, MO, USA). Glucose, blood urea nitrogen (BUN) and creatinine kits were purchased from Accurex (Mumbai, India). ELISA kits for ACE, ACE2, Ang-(1–7) and Ang II were obtained from Fn Test (Wuhan, China). ACE, ACE2, AT1R, AT2R, and monocytes chemoattractant protein (MCP-1) primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and the remaining primary and secondary antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA).

2.2. Development of streptozotocin-induced type 1 diabetes

The animal experiments were performed at Central Animal Facility (CAF), Birla Institute of Technology and Science Pilani (BITS-Pilani) as per the protocol approved by the Institutional Animal Ethics Committee (IAEC), BITS-Pilani (Protocol Approval No: IAEC/RES/21/08). Animal studies are reported ensuing the ARRIVE guidelines [14]. The male adult Wistar rats (200-220 g) were procured from the CAF of BITS-Pilani and were maintained under standard environmental conditions, with feed and water ad lib. Diabetes was induced by injecting a single dose of streptozotocin [55 mg/kg, *i.p.*, vehicle- sodium citrate buffer (0.01 M, pH 4.4)] in male Wistar rats, as described previously [15]. ND rats with same age group received only sodium citrate buffer ($n = 40$). After 48 h of streptozotocin injection, rats showing plasma glucose levels > 16 mmol/L were included in the study as DM rats ($n = 40$).

2.3. Ischemic renal injury in non-diabetic and diabetes mellitus rats

ND and DM rats were injected with saline (20 ml/kg, *s.c.*) to prevent fluid loss during laparotomy. Rats were anaesthetised with pentobarbital sodium (50 mg/kg, *i.p.*) and kept on a homoeothermic blanket to maintain body temperature (37 °C). After the loss of pedal pain and corneal reflexes, a half-inch incision was given on the left flank portion of abdomen and kidney was pulled out of the abdomen by holding the perirenal fat at the lower pole with blunt forceps. Followed by the clamping of renal vascular pedicle with a surgical clamp to induce ischemia [16]. After 45 min, the clamps were released, and 48 h of reperfusion was done. Then skeletal muscle and skin layers were sutured separately with absorbable and non-absorbable sutures, respectively. After suturing, topical (Betadine™) antiseptic and parenteral (Augmentin™, 324 mg/kg, *i.p.*) antibiotics were given to prevent post-surgical infection. Sham control animals were subjected to identical operation without renal vascular pedicle clamping. After 48 h of reperfusion; the rats were again anaesthetised with pentobarbital sodium (50 mg/kg, *i.p.*). Blood samples were collected from vena cava with a 5 mL syringe and plasma were separated (centrifugation) for biochemical parameters like plasma glucose (PGl), blood urea nitrogen (BUN), plasma creatinine (PCr), and ELISA. Th left kidney (I/R kidney) was then removed, washed and blotted dry, weighed and kept in −80 °C. Further, the left kidney was taken for tubular fraction isolation, western blot, ELISA, immunohistochemistry (IHC) and histological examinations.

2.4. Treatment regimens

Both ND and DM rats were subdivided into five groups each: (i) **ND/DM-** serve as respective controls, (ii) **ND − /DM-I/R-** ND or DM rats subjected to Ischemia-45 min/reperfusion-48 h (I/R), (iii) **ND − /DM-I/R + C21–** ND-I/R or DM-I/R rats receiving compound 21 (C21) (0.3 mg/kg/day, *i.p.*) [17], (iv) **ND − /DM-I/R + Dize-** ND-I/R or DM-I/R rats receiving diminazene aceturate (Dize) (5 mg/kg/day, *p.o.*) [9], (v) **ND − /DM-I/R + CD-** ND-I/R or DM-I/R rats receiving C21 (0.3 mg/kg/day, *i.p.*) and Dize (5 mg/kg/day, *p.o.)* combination therapy (Fig. 1A–B). We kept six rats in each experimental group considering an effect size of 0.92, α of 0.05, and power of 0.95 for statistical analysis.

2.5. Proximal tubules isolation from the whole kidney

Collected kidneys were placed in cold PBS (pH- 7.4), and tubular fractions were isolated using the percoll gradient centrifugation method with some modifications [18]. Briefly, the kidney was minced and digested with collagenase type IV in PBS, with constant oxygenation until a uniform suspension was formed. The suspension was filtered through a nylon 250-μm sieve and centrifuged at 100 *g* for 1 min. The pellets were suspended and washed two times in ice-cold PBS. The pellet suspension in PBS was mixed thoroughly with 40% Percoll and centrifuged at 26,000 *g* for 30 min. Four distinct bands (B1-B4) were separated. The B4 band, highly enriched proximal tubular fraction, was carefully collected, suspended, and washed in ice-cold PBS. Thus, the obtained tubular fraction was assessed under the light microscope and used for further analysis.

2.6. Histopathology

Renal histology was examined by Hematoxylin and Eosin (H and E) staining [19]. At least 4–5 sections (one microscopy slide) from each kidney and a total of $n = 6$ kidneys from each group were observed; and images of cortical tubules were captured at 400× magnification by using a Zeiss microscope (model: Vert.A1). 5–6 images from each stained kidney microscopy slide were evaluated for tubular necrosis. The histological findings were semi-quantitatively scored from 0 to 3 by a blinded observer: 0, none; 1, < 25% of tubules affected; 2, 25–50% of tubules affected; 3, > 50% of tubules affected. The average value of tubular necrosis score for each kidney was considered for statistical analysis ($n = 6$ kidneys/group).

2.7. Immunohistochemistry

IHC was performed as described previously [9]. Briefly, kidney sections (5 μm) were taken from paraffin blocks and deparaffinized with xylene, followed by antigen retrieval by heating in citrate buffer (10 mmol/L). We have used rabbit/mouse monoclonal antibodies against, ACE, ACE2, AT1R, and AT2R (Dilution: 1:200 *v*/v) as primary antibodies, and HRP-conjugated anti-rabbit/mouse IgG as a secondary antibody. Followed by detection with diaminobenzidine (DAB) as a chromogen. The slides were counterstained with hematoxylin, dehydrated with alcohol and xylene and mounted in DPX. At least 4–5 sections (one microscopy slide) from each kidney and a total of $n = 6$ kidneys from each group were observed; and images were captured at 400× magnification by using Zeiss microscope (model: Vert.A1). 5–6

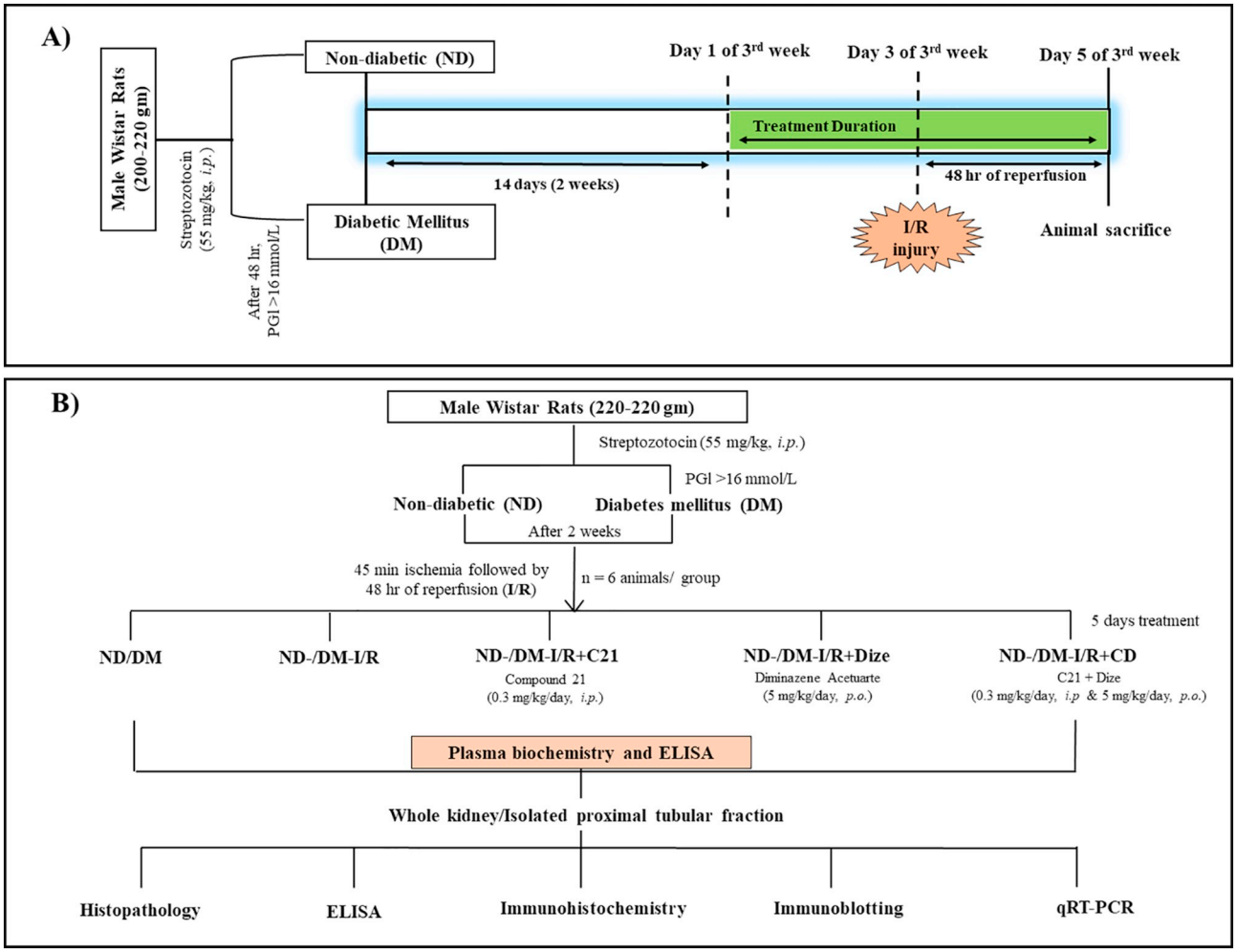

Fig. 1. Schematic representation of the interventional study.
A) Schedule for the induction of diabetes mellitus by streptozotocin-injection and acute kidney injury (AKI) by ischemia/reperfusion (I/Rin), and pharmacological intervention. B) Details of animal sub-grouping for an intervention study and further experimental design ($n = 6$ animals/group). **ND**- non-diabetic rats, **DM**-diabetes mellitus rats, **ND − /DM-I/R**- ND or DM rats subjected to Ischemia-45 min/reperfusion-48 h (I/R), **ND − /DM-I/R + C21–** ND-I/R or DM-I/R rats receiving compound 21 (0.3 mg/kg/day, *i.p.*) monotherapy, **ND − /DM-I/R + Dize-** ND-I/R or DM-I/R rats receiving Dize (5 mg/kg/day, *p.o.*) monotherapy, **ND − /DM-I/R + CD-** ND-I/R or DM-I/R rats receiving compound 21 (0.3 mg/kg/day, *i.p.*) and Dize (5 mg/kg/day, *p.o.)* combination therapy.

images from each stained kidney microscopy slide were analysed using ImageJ software (NIH, Bethesda, MD, USA) for calculating DAB-positive area.

2.8. Elisa

We homogenised isolated proximal tubular fraction in the recommended buffer solution, followed by total protein estimation by Lowry's method. Protein equalised proximal tubular samples, and diluted plasma were assayed for ACE, ACE2, Ang II and Ang-(1–7) protein levels by using ELISA kits ($n = 6$ rats/group) [9].

2.9. Immunoblotting

Protein isolation and immunoblotting were performed as previously described [9]. For immunoblotting, we have used rabbit/mouse/goat monoclonal antibodies against; p-NF-κB, c-Caspase-3, c-PARP1, Nrf2, MCP-1 and β-actin [Dilution 1:1000 (*v*/v)] as primary antibody and HRP conjugated anti-rabbit/mouse/goat IgG as secondary antibody [Dilution 1:20000 (v/v)]. Proteins were detected by using the ECL system and Hyperfilm, subsequently quantified by densitometric measurements using ImageJ software. The exposures were in linear dynamic range. Data analysis was performed by using GraphPad Prism software (San Diego, CA, USA), and results were expressed as fold change over control.

2.10. Quantitative real-time polymerase chain reaction

RNA was isolated from proximal tubular fraction using commercially available kits and qRT-PCR were performed using specific primers (Supplementary data, Table S1), designed and produced by Eurofins, India. [9,15]. Isolated RNA was reverse transcribed. qRT-PCR was done with LightCycler® 96 Real-Time PCR System using the FastStart Essential DNA Green Master and results were analysed by LightCycler® Software (Roche, Germany). Enrichment of targeted mRNA was normalized against 18s rRNA contents. Experiments were carried out in triplicate for each sample and results are expressed as fold changes over respective controls.

2.11. Statistical analysis

Experimental values are represented as mean ± SD, and 'n' refers to the number of samples studied. Statistical comparison between different groups was performed using one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post hoc test. Data were considered statistically significant if $p < 0.05$. GraphPad Prism

Table 1
Plasma biochemical parameters. Nondiabetic (ND) and diabetes mellitus (DM) (after 2 weeks of STZ-injection) rats were subjected to ischemic- 45 min and reperfusion of 48 h (I/R), followed by metabolic parameter measurement-plasma glucose (PGl), blood urea nitrogen (BUN), plasma creatinine (PCr).

Parameters	ND	ND-I/R	DM	DM-I/R
PGl (mmol/L)	4.9 ± 0.18	4.9 ± 0.42	16.9 ± 1.04[a]	17.3 ± 1.29[ab]
BUN (mmol/L)	7.1 ± 0.71	9.8 ± 0.53[a]	7.3 ± 0.49	13.8 ± 0.66[a,cb]
PCr (mg/dL)	1.56 ± 0.12	1.59 ± 0.16	1.57 ± 0.19	1.62 ± 0.11

Note: Each data is represented as mean ± SD (n = 6).
[a] $P < 0.05$ vs. ND.
[b] $P < 0.05$ vs. ND-I/R.
[c] $P < 0.05$ vs. DM.

software version 7.00 (San Diego, CA, USA) was used for all statistical processing.

3. Results

3.1. Hyperglycaemia increases the severity of ischemic renal injury

To check the effect of hyperglycaemia on IRI, we subjected ND and DM rats to ischemia followed by reperfusion (I/R) and evaluated the plasma biochemistry and extent of the oxidative stress markers in the isolated proximal tubular fraction. We observed that IRI in ND and DM rats significantly increased BUN levels when compared to respective controls. Interestingly, DM-I/R exhibited augmented BUN levels in comparison to ND-I/R rats. No change was observed in plasma creatinine levels among all the study groups (Table 1). Moreover, IRI in ND and DM rats significantly increased renal tubular oxidative stress, as demonstrated by augmented malondialdehyde (MDA) and GSH levels, nitrate/nitrite (NO_2/NO_3) ratio, and catalase activity compared to respective controls. Proximal tubules isolated from IR kidneys of DM rats showed significantly higher oxidative stress as compared to ND rats (Fig. 2A–D). Thus, our data indicated IRI hypersensitivity in diabetic rats.

Next, we analysed the expression of proinflammatory cytokines and apoptosis markers by western blot in I/R kidneys from ND and DM rats. IRI augmented tubular inflammation as evinced by increased p-NF-κB and MCP-1 expressions, as well as caused proximal tubular cell apoptosis as demonstrated by increased c-PARP1 and c-Cas-3 expressions when compared to respective controls (Fig. 2E–I). Interestingly, expressions of p-NF-κB, MCP-1, c-PARP1, and c-Cas-3 were significantly higher in I/R kidneys of DM as compared to ND rats (Fig. 2E–I).

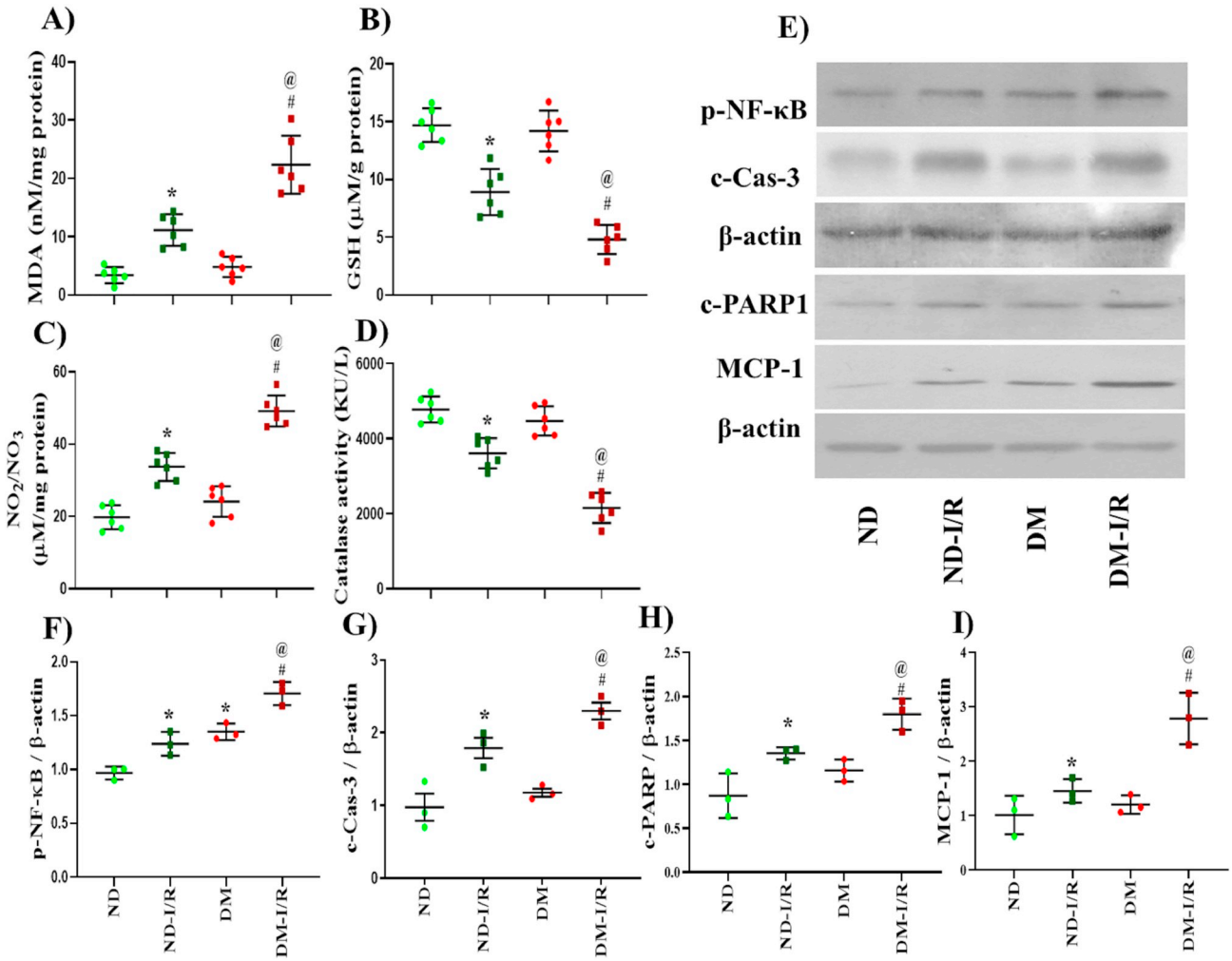

Fig. 2. Evaluation of oxidative stress, inflammation and apoptosis in IRI.
A-D: Estimation of oxidative stress markers e.g. malondialdehyde (MDA) (A), GSH (B), NO_2/NO_3 (Griess) (C), catalase activity (D) in kidneys of rats. ($n = 6$). *E*-I: Immunoblots for protein expressions of inflammatory and apoptotic markers in the isolated renal proximal tubular fraction with β-actin as a loading control (E). Immunoblots were quantified by densitometry analysis e.g. NF-κB(S-536) (F), c-Caspase-3 (G), c-PARP1 (H), and MCP-1 (I). Data are represented as mean ± SD from three independent experiments. For statistical comparison, one-way ANOVA with Tukey's multiple comparison test was used where (*) $p < 0.05$ vs ND; (#) $p < 0.05$ vs DM; (@) $p < 0.05$ vs ND-I/R.

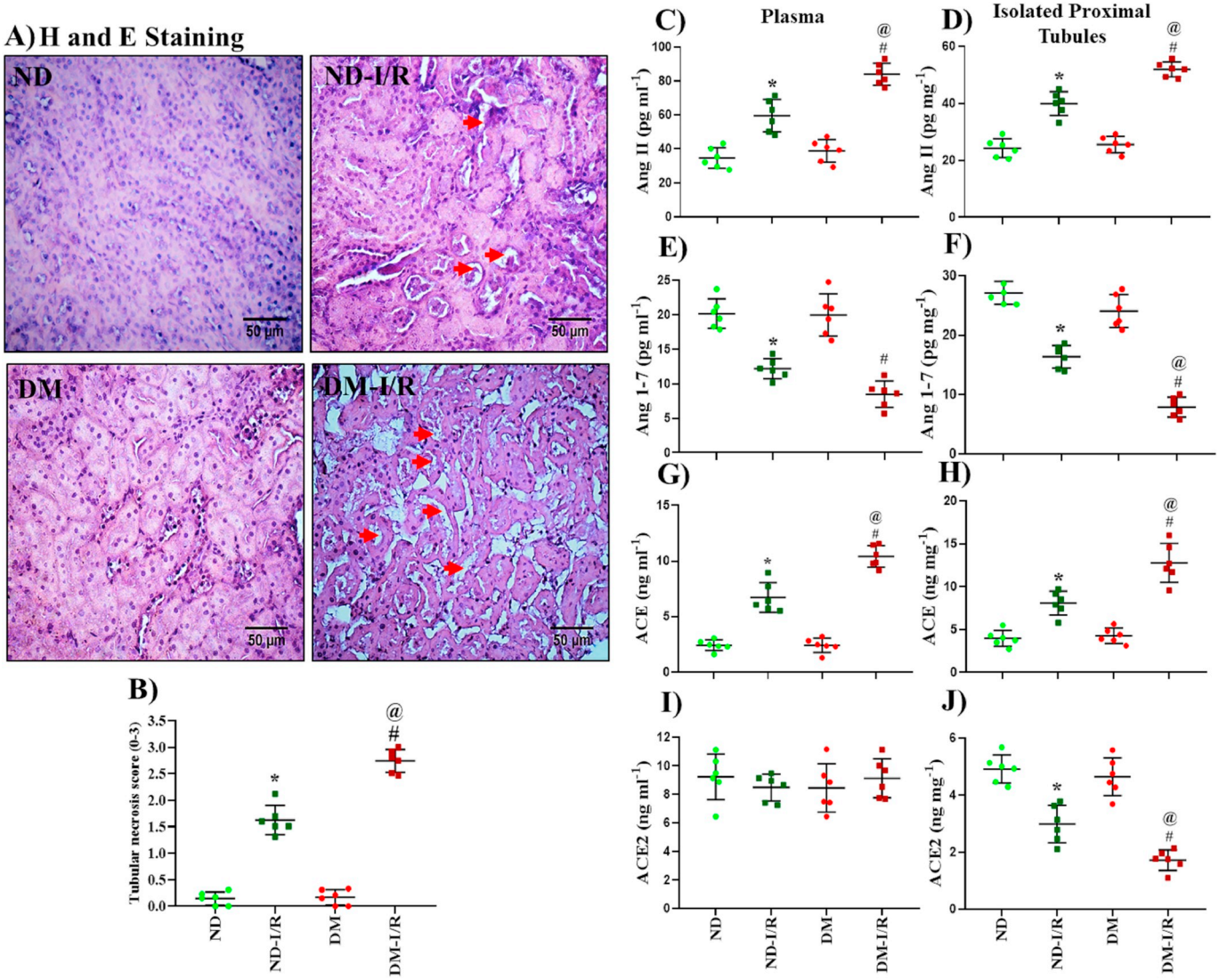

Fig. 3. IRI modulates tubular necrosis, and systemic and proximal tubular specific ACE, ACE2, Ang II and Ang-(1–7) levels.
A-B: Representative H & E staining images of the cortical region of kidney transverse sections (original magnification 400× and scale bar- 50 µm). At least 4–5 images from each stained kidney section and a total of six different kidneys per group were observed by a blinded observer for tubular necrosis (arrow) (A). The tubular necrosis was analysed semi-quantitatively and scored from 0 to 3 (B). C-J: Protein expression of ACE, ACE2, Ang II and Ang-(1–7) in plasma (C, E, G, I) and isolated proximal tubules (D, F, H, J) was measured by ELISA ($n = 6$). Data are represented as mean ± SD. For statistical comparison, one-way ANOVA with Tukey's multiple comparison test was used where (*) $p < 0.05$ vs ND; (#) $p < 0.05$ vs DM; (@) $p < 0.05$ vs ND-I/R.

Furthermore, we also examined the histopathological alterations persuaded by unilateral renal ischemia in ND and DM rats by H and E staining. Tubular necrosis was observed in the outer cortex of DM-I/R rats and ND-I/R rats; however, DM-I/R rats showed extensive necrosis compared to ND-I/R rats (Fig. 3A–B). These results further confirm the higher susceptibility of diabetic kidney to I/R-induced AKI.

3.2. Ischemic renal injury alters systemic and tissue-specific renin-angiotensin system components in non-diabetic and diabetes mellitus rats

We measured the Ang II, Ang-(1–7) ACE, and ACE2 levels in plasma and proximal tubular fraction by ELISA kits (Fig. 3C–J). ND-I/R and DM-I/R rats demonstrated increased Ang II and decreased Ang-(1–7) levels in plasma and isolated tubules when compared to ND and DM rats, correspondingly (Fig. 3C–F). Furthermore, IRI to ND and DM rats increased ACE levels in plasma and isolated tubules, whereas decreased ACE2 levels only in isolated tubules with no change in plasma ACE2 levels (Fig. 3G–J). Interestingly, like our previous observations, IRI significantly altered the RAS components levels in the DM rats when compared to ND rats. Furthermore, IHC revealed increased ACE with no change in ACE2 expressions in I/R kidneys of ND as compared to control rats, whereas I/R kidneys of DM rats demonstrated increased ACE and reduced ACE2 tubular expression in comparison to DM and ND-I/R rats (Fig. 4A–B, E–F). In contrast, renal expression of AT1R and AT2R were significantly augmented in kidneys after IRI as compared to respective controls (Fig. 4C–D, G–H). Moreover, DM-I/R rats' kidneys exhibited increased tubular AT1R expression in comparison to ND-I/R rats' kidneys (Fig. 4C and G). Together our data suggest that the IRI altered the expression of the RAS depressor arm's components in both DM and ND conditions.

3.3. AT2R and ACE2 activation improved renal functions, inhibited renal oxidative stress and apoptosis in non-diabetic and diabetes mellitus rats upon ischemic renal injury

Based on our previous results we urged to check the role of RAS depressor arm's modulations on IRI, thus we treated ND-I/R and DM-I/R rats with AT2R agonist (C21, 0.3 mg/kg/day, *i.p.*) and ACE2 activator

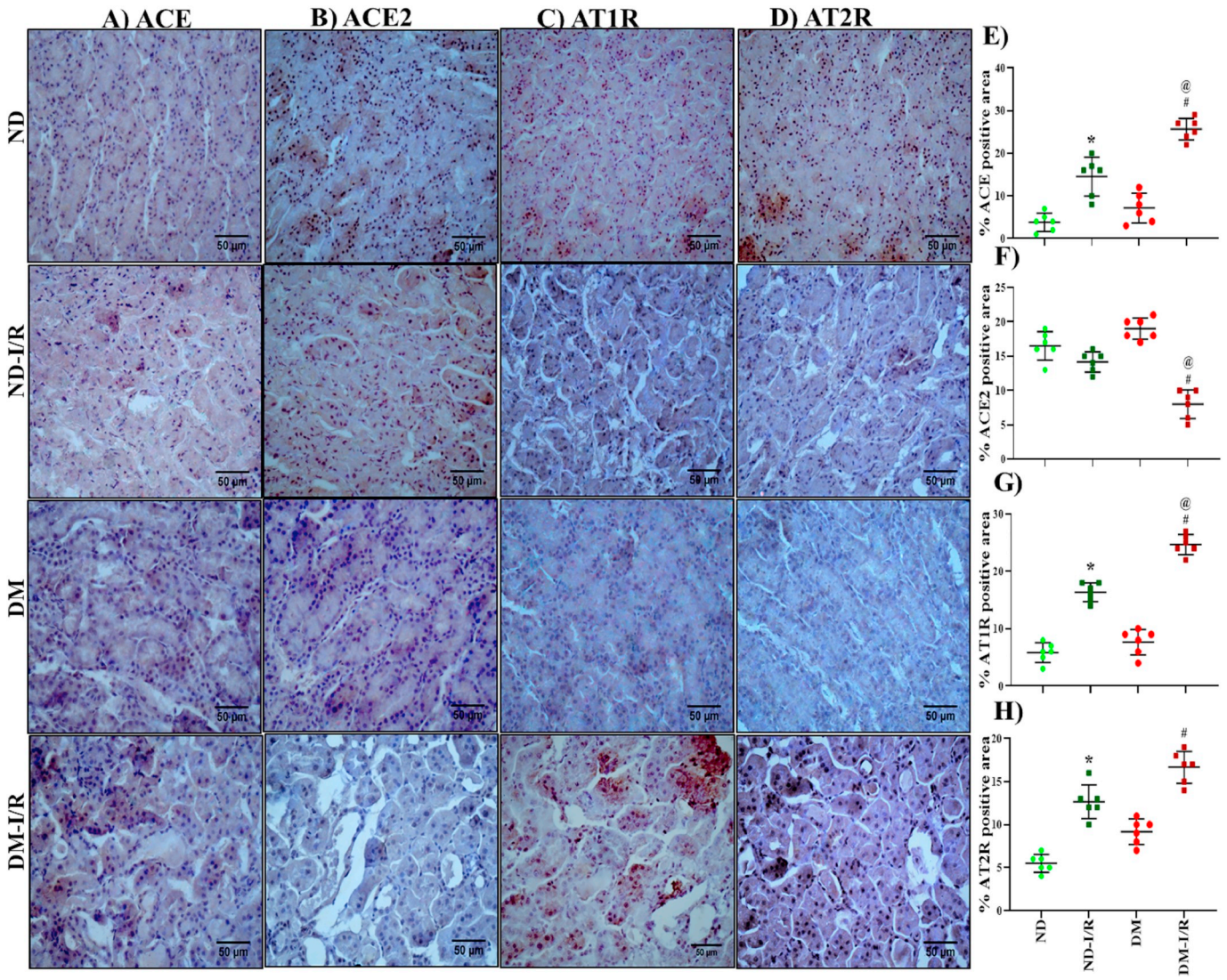

Fig. 4. Immunohistochemistry for ACE, ACE2, AT1R and AT2R.
A-D: Representative images of IHC staining for ACE, ACE2, AT1R and AT2R in the cortex of kidney (original magnification 400× and scale bar 50 µm). At least 4–5 sections from each stained kidney microscopy slide and total six different kidney slides per group were observed under microscope and images were captured. E-H: Semi-quantitative analysis of all the captured images using ImageJ (colour deconvolution plugin was utilized for analysis) for calculating DAB-positive area (indicates specific protein expressions). All Data are represented as mean ± SD. One-way ANOVA with Tukey's multiple comparison test, where (*) $p < 0.05$ vs ND; (#) $p < 0.05$ vs DM; (@) $p < 0.05$ vs ND-I/R.

Table 2
Plasma metabolic parameters: At the end of the treatment period, we have performed plasma biochemistry by assaying plasma glucose (PGL), blood urea nitrogen (BUN) levels in all the experimental groups.

Parameters	ND	ND-I/R	ND-I/R + C21	ND-I/R + Dize	ND-I/R + CD	DM	DM-I/R	DM-I/R + C21	DM-I/R + Dize	DM-I/R + CD
PGL (mmol/L)	6.1 ± 0.8	6.3 ± 1.0	5.9 ± 0.7	6.9 ± 0.9	6.6 ± 0.6	20.1 ± 1.7	19.3 ± 1.2	19.8 ± 2.0	18.5 ± 2.4	19.9 ± 0.9
BUN (mmol/L)	6.8 ± 0.3	9.2 ± 0.5[a]	9.8 ± 0.6	9.5 ± 0.5	7.6 ± 0.4[b]	7.5 ± 0.8	13.3 ± 0.4[a,c]	12.1 ± 0.5	11.8 ± 0.6	8.7 ± 0.4[d]

Note: Each data is represented as mean ± SD (n = 6).
[a] $P < 0.05$ vs. ND.
[b] $P < 0.05$ vs. ND-I/R.
[c] $P < 0.05$ vs. DM.
[d] $P < 0.05$ vs.DM-I/R.

(Dize, 5 mg/kg/day, *p.o.*), either alone as monotherapy or together as combination therapy. Plasma biochemistry revealed that, AT2R agonist and ACE2 activator combination therapy scientifically lower the BUN levels, whereas monotherapies had no effect on BUN levels in ND-I/R and DM-I/R rats (Table 2). C21 and Dize monotherapies to ND-I/R rats reduced MDA and increased Nrf2 expression, with no change in GSH levels. In contrast, both monotherapies could only increase Nrf2 expression in DM-I/R rats and had no effect on MDA and GSH levels. Interestingly, combination therapy significantly reduced MDA and increased GSH and Nrf2 levels in both ND-I/R and DM-I/R rats (Fig. 5A–D). Next, we checked the expression of apoptosis markers, c-Cas-3 and c-PARP1 by western blot. We found that combination therapy

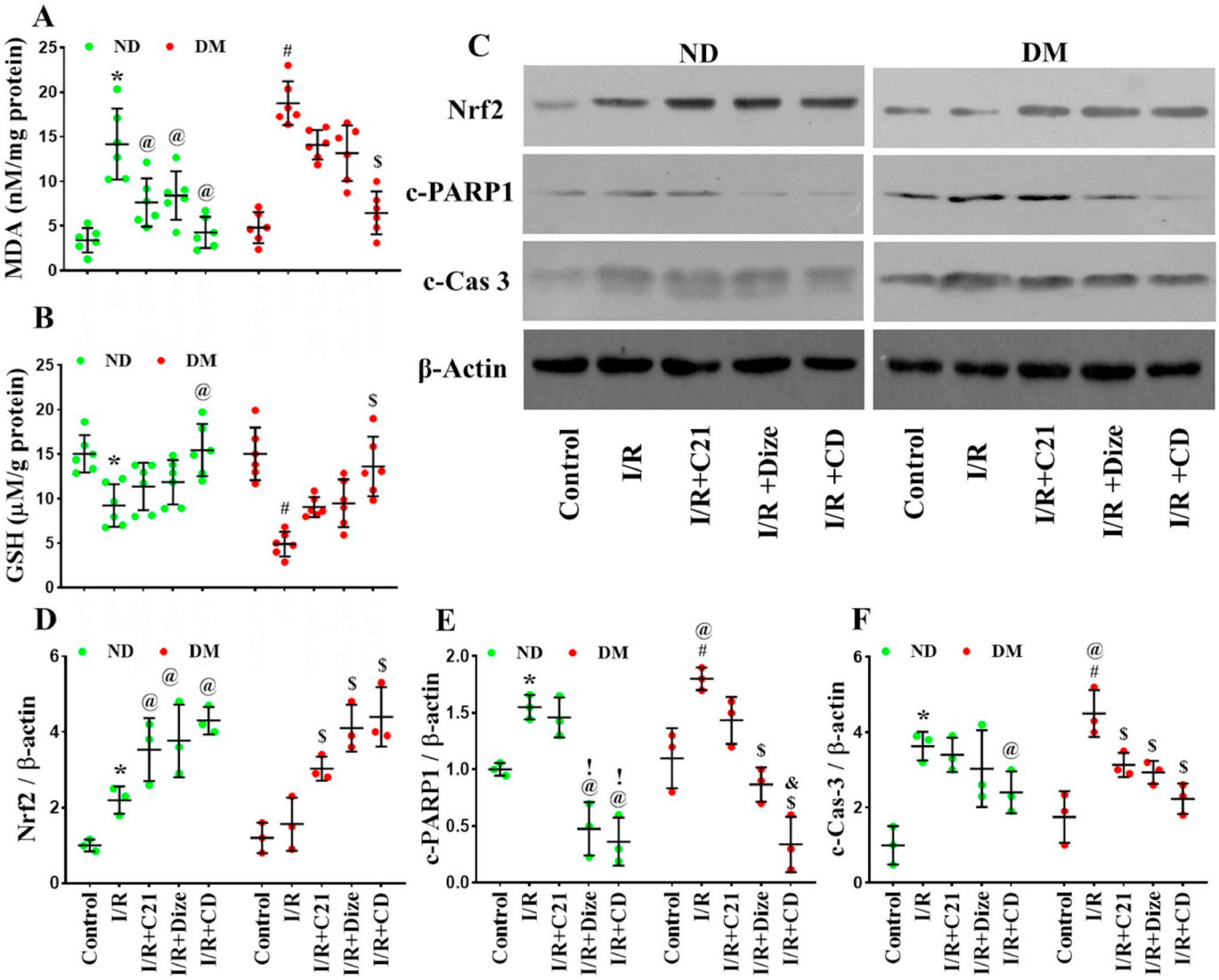

Fig. 5. AT2R agonist and ACE2 activator protects ND and DM rats against oxidative stress and apoptosis.
A-B: Estimation of oxidative stress markers e.g. malondialdehyde (MDA) (A), GSH (B) in proximal tubular fraction of kidneys of rats ($n = 6$). C-F: Representative western blot images for Nrf2, c-PARP1, c-Cas-3, and β-actin (loading control) protein expressions in isolated proximal tubular fraction (C). Immunoblots were quantified by densitometry analysis e.g. Nrf2 (D), c-PARP1 (E), and c-Cas-3 (F). Data are represented as mean ± SD from three independent experiments. One-way ANOVA with Tukey's multiple comparison test for statistical comparison. (*) $p < 0.05$ vs ND; (@) $p < 0.05$ vs ND-I/R; (#) $p < 0.05$ vs DM; ($) $p < 0.05$ vs DM-I/R; (&) $p < 0.05$ vs DM-I/R + C21.

significantly reduced c-Cas-3 and c-PARP1 levels in I/R kidneys of both ND and DM rats (Fig. 5C, E–F).

3.4. AT2R and ACE2 activation inhibits renal inflammation and prevented tubular damage in non-diabetic and diabetes mellitus rats upon ischemic renal injury

Inflammation is hallmark for the IRI [20], thus we analysed the alteration in the expressions of inflammatory molecules in the tubular fraction of I/R kidneys after AT2R and ACE2 activation. We observed that IRI increased protein expressions of p-NF-κB and MCP-1 in proximal tubules of ND and DM rats signifying renal inflammation. C21 and Dize monotherapies did not change the expression of p-NF-κB and MCP-1; except Dize monotherapy reduced MCP-1 expression in DM-I/R rats. In contrast, the combination therapy significantly inhibited p-NF-κB and MCP-1 expressions in the tubular fractions of both ND-I/R and DM-I/R rats (Fig. 6A–C). Furthermore, we found a significant increase in mRNA expressions of interleukin-6 (*Il6*), tumour necrosis factor-α (*Tnfa*) and *Mcp1* in the tubular fraction of ND and DM rats subjected to IRI (Fig. 6D–F). C21 or Dize monotherapy did not change mRNA expressions of *Tnfa* and *Mcp1* in ND-I/R and DM-I/R rats, whereas reduced *Il6* mRNA expression only in DM-I/R rats. In contrast, combination therapy significantly reduced *Il6* and *Mcp1* mRNA expressions in ND-I/R and DM-I/R rats, while decreased *Tnfa* mRNA expression in ND-I/R with no change in *Tnfa* mRNA expression in DM-I/R rats (Fig. 6D–F).

Next, we performed histopathological evaluation of kidney cortex by H and E staining. ND-I/R and DM-I/R rats exhibited significantly increased tubular necrosis, which was not prevented by C21 and Dize monotherapies. However, the combination therapy produced marked reduction in tubular necrosis in ischemic ND and DM kidneys' (Fig. 7). Therefore, our data suggest that simultaneous activation of AT2R and ACE2 inhibits renal inflammation and tubular damage in ND and DM rats upon IRI.

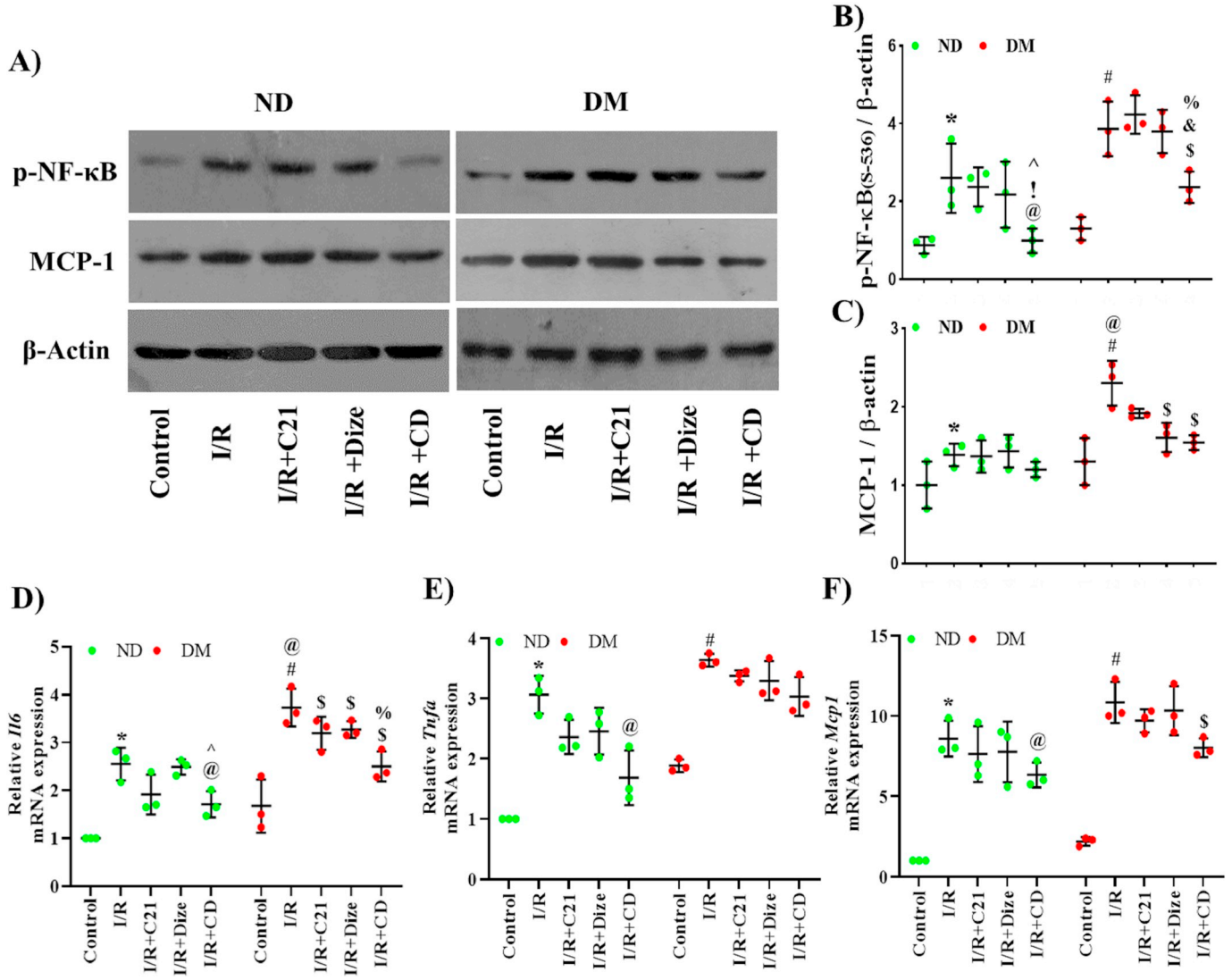

Fig. 6. Effect of C21, Dize and their combination therapy on protein and mRNA expressions of inflammatory markers.
A-C: Representative western blot images for protein expressions of p-NF-κB, MCP-1 and β-actin (loading control in isolated proximal tubular fraction (A). Immunoblots were quantified by densitometry analysis e.g. p-NF-κB (B), and MCP-1 (C). D-F: mRNA expression of *Il6, Tnfa* and *Mcp1* was assessed by qRT-PCR in isolated proximal tubules. 18s rRNA expression was used as internal control. Data are represented as mean ± SD from three independent experiments. One-way ANOVA with Tukey's multiple comparison test was used for statistical comparison. (*) $p < 0.05$ vs ND; (@) $p < 0.05$ vs ND-I/R; (!) $p < 0.05$ vs ND-I/R + C21; (^) $p < 0.05$ vs ND-I/R + Dize; (#) $p < 0.05$ vs DM; ($) $p < 0.05$ vs DM-I/R; (&) $p < 0.05$ vs DM-I/R + C21; (%) $p < 0.05$ vs DM-I/R + Dize.

3.5. AT2R and ACE2 activation restores the altered systemic and tissue-specific RAS components in non-diabetic and diabetes mellitus rats upon ischemic renal injury

According to our previous results, IRI altered the systemic and tissue-specific RAS components in ND and DM rats. Therefore, next we checked the effect of AT2R and ACE2 activator treatments on the same. All three-treatment regimen increased tubular ACE2 levels in ND-I/R and DM-I/R rats and reduced plasma ACE levels in DM-I/R rats, while only combination therapy reduced plasma ACE level in ND-I/R rats (Fig. 8A and F). None of the treatment regimens could alter the plasma ACE2 and tubular ACE levels in ND-I/R and DM-I/R rats (Fig. 8B and E). In plasma, Ang II and Ang-(1–7) levels remain unchanged after C21 monotherapy, while Ang-(1–7) levels significantly increased after Dize monotherapy in ND-I/R and DM-I/R rats. Interestingly, combination therapy significantly reduced Ang II levels and increased Ang-(1–7) levels in plasma of ND-I/R and DM-I/R rats (Fig. 8C–D). In isolated tubular fraction, all three-treatment regimen significantly reduced Ang II and augmented Ang-(1–7) levels in ND-I/R and DM-I/R rats; except C21 monotherapy did not change tubular Ang II and Ang-(1–7) levels in ND-I/R rats (Fig. 8G–H). One of the consistent features is that combination therapy was better in normalizing the RAS components levels when compared to respective monotherapies.

Next, we checked mRNA expressions of *At1r, At2r* and *Masr* in a tubular fraction using qRT-PCR. ND-I/R and DM-I/R rats exhibited increased mRNA expressions of *At1r, At2r* and *Masr* in a tubular fraction when compared to respective controls. C21 or Dize monotherapy did not change mRNA expressions of *At1r, At2r* and *Masr* in ND-I/R and DM-I/R rats. Interestingly, the combination therapy resulted in a further increase in *At2r* and *Masr* mRNA expressions, with no change in *At1r* mRNA expressions in ND-I/R and DM-I/R rats (Fig. 8I–K). Therefore, our data suggest that the protective effects seen after the combination therapy might relates to the restoration of systemic and tissue-specific RAS components in ND and DM rat upon IRI.

4. Discussion

We hypothesised that the depressor arm of RAS plays a major role in

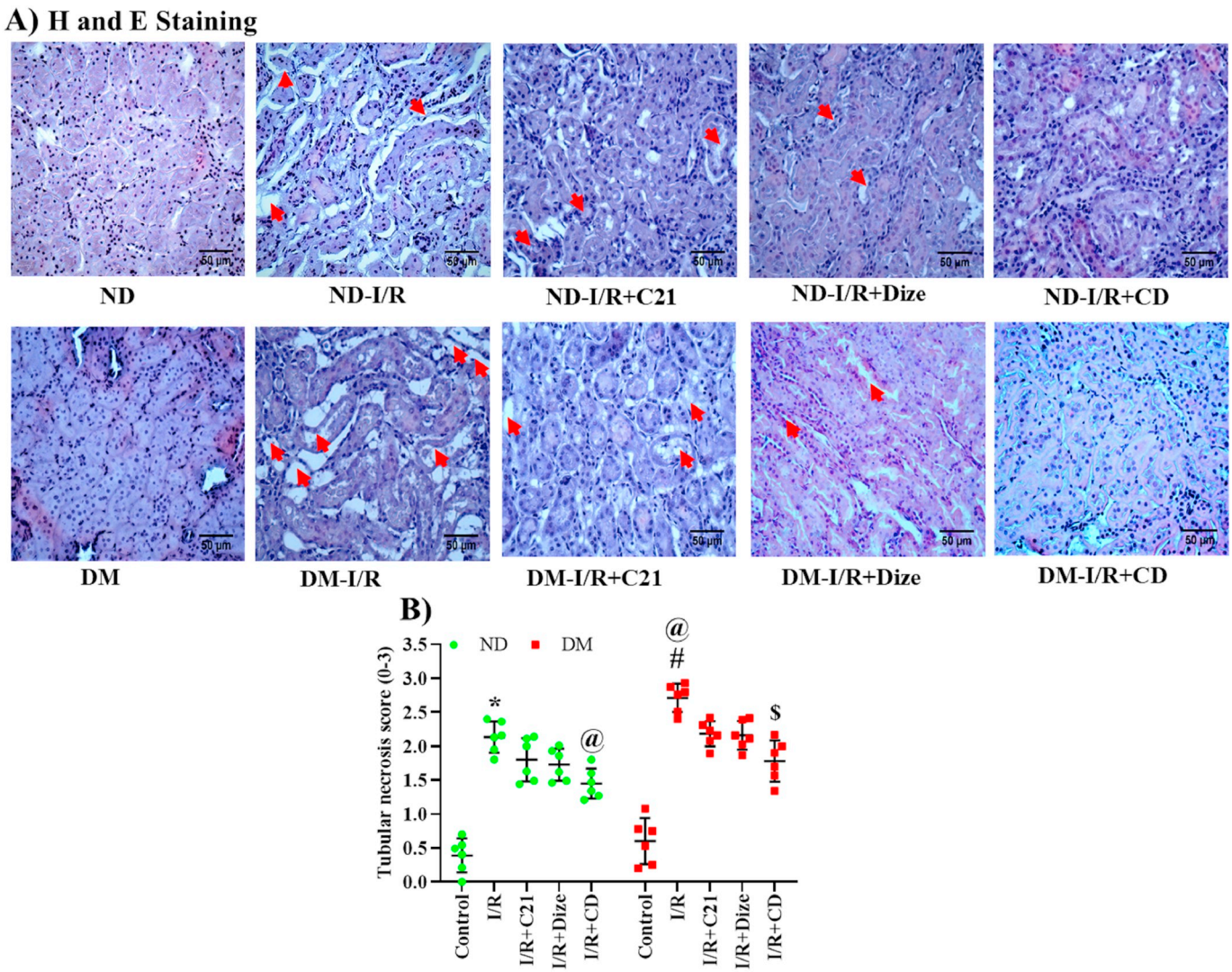

Fig. 7. AT2R agonist and ACE2 activator prevented tubular necrosis associated with IRI.
Representative images for H and E staining of kidney sections (original magnification 400× and scale bar- 50 µm). At least 4–5 images from each stained kidney section and a total of six different kidneys per group were observed by a blinded observer for tubular necrosis (Red arrow) (A). The tubular necrosis was analysed semi-quantitatively and scored from 0 to 3 (B). Data are represented as mean ± SD. One-way ANOVA with Tukey's multiple comparison test was applied for statistical comparison. (*) $p < 0.05$ vs ND; (@) $p < 0.05$ vs ND-I/R; (#) $p < 0.05$ vs DM; ($) $p < 0.05$ vs DM-I/R; (&) $p < 0.05$ vs DM-I/R + C21. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

IRI under both diabetic and non-diabetic conditions. Our result demonstrated augmented AT1R, ACE, and Ang-II expressions, as well as AT2R and reduced ACE2 and Ang-(1–7) expression in proximal renal tubules of DM and ND rats subjected to IRI. Interestingly, administration of AT2R agonist (C21) and ACE2 activator (Dize) per se marginally ameliorated pathological changes associated with IRI including metabolic perturbation, increased renal tubular cells oxidative stress, apoptosis, and inflammation. However, their combination therapy significantly normalized the alterations in RAS components, thereby attenuated above-mentioned pathological consequences associated with IRI in diabetic and non-diabetic rats.

The epidemiologic reports advocated that AKI is more lethal in diabetic patients in comparison to non-diabetic individuals; however, responsible molecular mechanisms are still elusive [5]. In the present study, two weeks after STZ-injection, DM rats were subjected to I/R to induce AKI, we used this experimental animal model to mimic the pathophysiology of comorbid diabetes and AKI in humans (Fig. 1). We observed that IRI increased BUN levels and oxidative stress in DM rats compared to ND rats (Table 1, Fig. 2). Existing literature has speculated that critical pathogenic factors of AKI comprises compromised kidney perfusion and altered the intrarenal hemodynamic balance, which largely attributed to systemic and intrarenal RAS activation [21]. Individually, hyperglycaemia or IRI increased levels of Ang II; an octapeptide and the major effector of RAS pressor arm mediating pathological effects via activation of AT1R [8,22]. Consistent with this, we observed activation of the pressor arm of the RAS demonstrated by increased Ang II, ACE and AT1R expression in a renal proximal tubular fraction from kidneys of diabetic rats that underwent IRI (Figs. 3, 4).

On the other hand, the depressor arm components: AT2R, ACE2, Ang-(1–7) are identified for their positive feedback mechanism by recognising elevated cellular stress and activated pathological signalling [9,10]. Male Wistar rats subjected to left nephrectomy and 45 min ischaemia on right kidney demonstrated reduced renal ACE2 mRNA expression and Ang-(1–7) levels at 4 h reperfusion [10]. In subtotal nephrectomised rats, Dize (ACE2 activator) augmented cortical and medullary ACE2 activity and abridged cortical ACE activity [23]. Recently, we have reported that Dize monotherapy (5 mg/kg/day) marginally attenuated diabetic renal fibrosis, whereas Dize in combination with a neprilysin inhibitor thiorphan significantly attenuated the development of diabetic nephropathy [24]. Further, C21 has

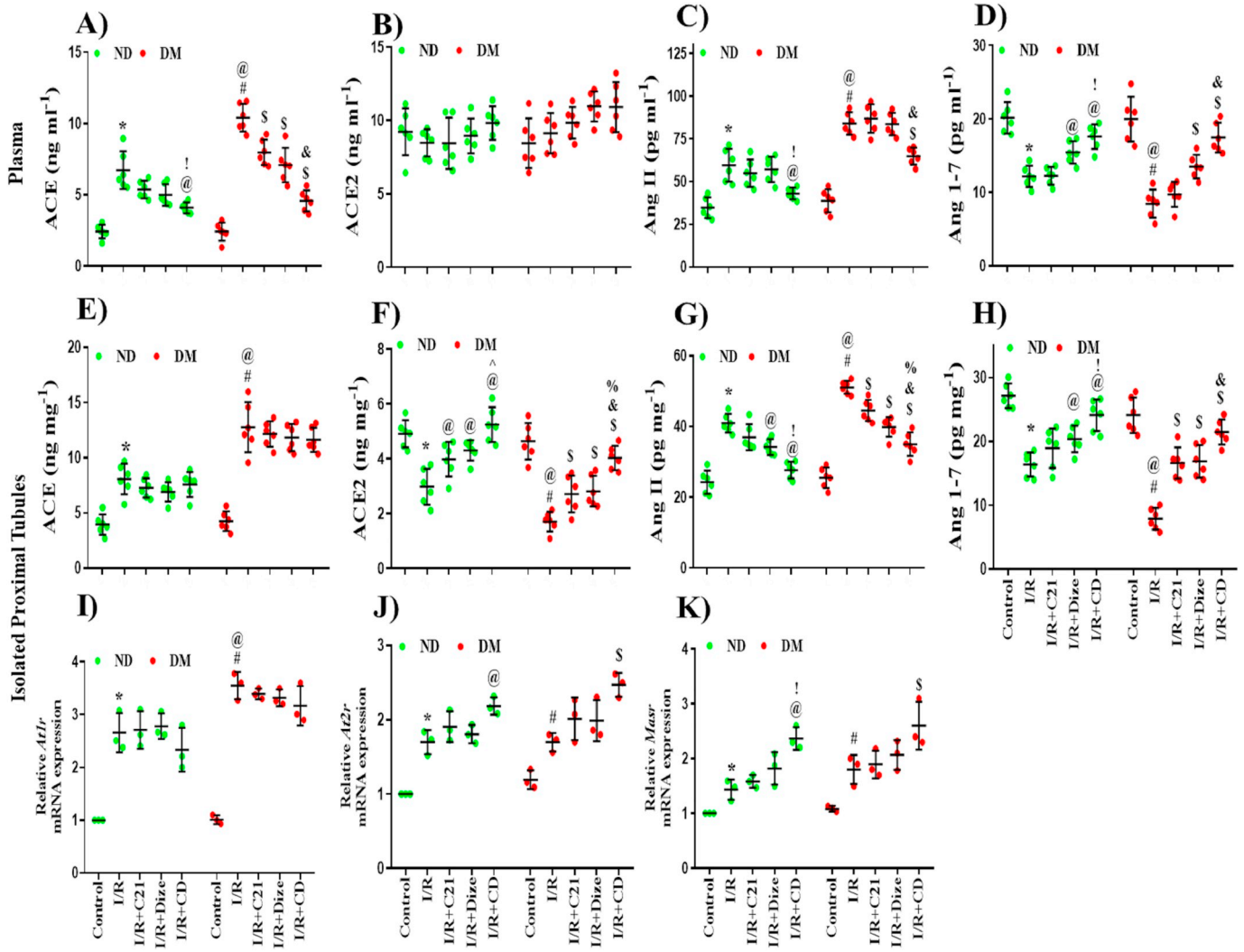

Fig. 8. Effect of C21, Dize and their combination therapy on protein and mRNA expressions of RAS components.
A-H: Protein expression of ACE, ACE2, Ang II and Ang-(1–7) in plasma (A-D) and isolated proximal tubules (D-J) was measured by ELISA (n = 6). I-K: mRNA expressions of *At1r*, *At2r* and *Masr* was assessed by qRT-PCR in isolated proximal tubules. 18s rRNA expression was used as internal control. Data are represented as mean ± SD from three independent experiments. One-way ANOVA with Tukey's multiple comparison test was applied for statistical comparison. (*) $p < 0.05$ vs ND; (@) $p < 0.05$ vs ND-I/R; (!) $p < 0.05$ vs ND-I/R + C21; (#) $p < 0.05$ vs DM; ($) $p < 0.05$ vs DM-I/R; (&) $p < 0.05$ vs DM-I/R + C21; (%) $p < 0.05$ vs DM-I/R + Dize.

demonstrated to halt the development of diabetic nephropathy in mice and rats [25,26]. Recently, we reported that C21 monotherapy at dose 0.3 mg/kg/day has partially improved renal functions, while C21 with Telmisartan combination has markedly mitigated diabetic nephropathy by attenuating apoptotic signalling [17].

In the present study, we observed reduced tubular ACE2 and Ang-(1–7) expressions and a compensatory increase in AT2R expression in DM-I/R rats when compared to DM and ND-I/R rats. Hence, we speculated that modulating RAS depressor arm by ACE2 activator or AT2R agonist might protect the kidney against IRI. On the basis of abovementioned reports, we treated ND-I/R and DM-I/R rats with AT2R agonist, C21 (0.3 mg/kg/day, *i.p.*) or ACE2 activator, Dize (5 mg/kg/day, *p.o.*) for five days. Our data suggest that the monotherapy using either AT2R agonist or ACE2 activator in ND-I/R and DM-I/R rats had only minor effect on tubular damage as evident from oxidative stress parameters and histopathological evaluations. Hence, we plan to administer AT2R agonist and ACE2 activator together as a combination therapy at same dose regimen in ND-I/R and DM-I/R rats. We found that concomitant AT2R agonism and ACE2 activation significantly attenuated oxidative stress associated with IRI in ND and DM rats (Fig. 5). Further, IRI has been reported to activate antioxidant transcription factor Nrf2 [27]. In this regard, we observed increased Nrf2 expression in renal tubular fraction of ND-I/R and DM-I/R rats. Interestingly, C21 and Dize combination therapy to ND-I/R and DM-I/R rats further amplified proximal tubular Nrf2 expression (Fig. 5). Interestingly, simultaneous AT2R agonist and ACE2 activator administration have attenuated proinflammatory cytokines *Il6, Tnfa* and *Mcp1* mRNA expressions and preventing NF-κB signalling-mediated inflammation, MCP-1-mediated leukocyte infiltration, and c-Cas-3 and cPARP-mediated apoptosis in the proximal tubular fraction of ND-I/R and DM-I/R rats (Figs. 5 and 6). Previous studies showed that C21 in combination with telmisartan or losartan alleviated glomerular damage, extracellular matrix accumulation, and increased glomerular nephrin expression in type 2 diabetic rats [17,28]. Similarly, we observed that C21, AT2R agonist along with Dize, ACE2 activator was better in mitigating morphological alterations (tubular necrosis) when compared to monotherapies (Fig. 7). Moreover, ELISA results revealed the superiority of C21 and Dize combination therapy over respective monotherapies in normalizing systemic (plasma) and local (proximal tubules) alteration in RAS components level [e.g. ACE, ACE2, Ang II and Ang-

(1–7)] associated with IRI in ND and DM rats (Fig. 8). The combination therapy further augmented mRNA expressions of renoprotective AT2R and MasR in IRI subjected ND and DM rats (Fig. 8). In brief, these results provide us with articulate evidence that AT2R and ACE2 are involved in the pathogenesis of ischemic AKI. In this study, elevated inflammatory, apoptotic and other pathogenic signalling can be correlated with dysregulated RAS depressor arm (reduced Ang-(1–7) and ACE2 levels) and severity of ischemic AKI. For the first time, we stated that the novel combination of AT2R agonist and ACE2 activator has significantly attenuated the IRI related kidney impairments in DM and ND rats.

5. Conclusion

To the best of our knowledge, this is the first report to revealed that suppression of the depressor arm of RAS precipitates ischemic AKI in ND and DM rats. However, the severity of ischemic AKI was found to be extensive in DM rats, which could be due to the presence of hyperglycaemia. The novel combination therapy of AT2R agonist and ACE2 activator targeting protective axis of RAS, significantly assuaged systemic and renal RAS alterations and prevented renal tubular damage associated with IRI in ND and DM rats. Thus, we suggested that targeting RAS depressor arm might serve as a novel therapeutic option against AKI in diabetic and non-diabetic conditions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116796.

Author contributions

A.B.G. and S.R.M conceived the idea and designed the experiments. N.S. and V.M. performed all the experiments and data analysis. All the authors were actively involved in manuscript writing.

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Declaration of competing interest

The authors declare no potential conflicts of interest.

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