Life Sciences 221 (2019) 159–167

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Concurrent neprilysin inhibition and renin-angiotensin system modulations prevented diabetic nephropathy

Vajir Malek, Nisha Sharma, Himanshu Sankrityayan, Anil Bhanudas Gaikwad*

Laboratory of Molecular Pharmacology, Department of Pharmacy, Birla Institute of Technology and Science Pilani, Pilani Campus, Rajasthan 333031, India

ARTICLE INFO

Keywords:
Diabetic nephropathy
Neprilysin inhibitor
Angiotensin receptor blocker
ACE2 activator
Thiorphan/telmisartan combination
Thiorphan/Dize combination

ABSTRACT

Aims: Renin-angiotensin system (RAS) and natriuretic peptides system (NPS) perturbations govern the development of diabetic nephropathy (DN). Hence, in search of a novel therapy against DN, present study targeted both, NPS and RAS simultaneously using a neprilysin inhibitor (NEPi) in combination with either angiotensin receptor blocker (ARB) or angiotensin-converting enzyme 2 (ACE2) activator.

Methods: We induced diabetes in male Wistar rats by a single dose of streptozotocin (55 mg/kg, *i.p.*). After four weeks, we treated diabetic rats with thiorphan, telmisartan or diminazene aceturate (Dize) 0.1, 10, 5 mg/kg/day, *p.o.* alone as monotherapy, or both thiorphan/telmisartan or thiorphan/Dize as combination therapy, for four weeks. Then, plasma and urine biochemistry were performed, and kidneys from all the groups were collected and processed separately for histopathology, ELISA and Western blotting.

Key findings: Proposed combination therapies attenuated metabolic perturbations, prevented renal functional decline, and normalised adverse alterations in renal ACE, ACE2, Ang-II, Ang-(1–7), neprilysin and cGMP levels in diabetic rats. Histopathological evaluation revealed a significant reduction in glomerular and tubulointerstitial fibrosis by combination therapies. Importantly, combination therapies inhibited inflammatory, profibrotic and apoptotic signalling, way better than respective monotherapies, in preventing DN.

Conclusion: Renoprotective potential of thiorphan (NEPi)/telmisartan (ARB) and thiorphan/Dize (ACE2 activator) combination therapies against the development of DN is primarily attributed to normalisation of RAS and NPS components and inhibition of pathological signalling related to inflammation, fibrosis, and apoptosis. Hence, we can conclude that NEPi/ARB and NEPi/ACE2 activator combination therapies might be new therapeutic strategies in preventing DN.

1. Introduction

Approximately 30% of diabetic individuals are affected by diabetic nephropathy (DN) and is one of the most common causes of the end-stage renal failure, globally [1–3]. Chronic hyperglycemia driven activation of the conventional renin-angiotensin system (RAS) plays a pivotal role in the development of DN. Consequently, therapeutic regimens aiming RAS blockage using angiotensin receptor blockers (ARBs) and angiotensin-converting enzyme inhibitor (ACEi) are beneficial in halting the progression of DN [1–3]. However, ARBs and ACEi are effective in delaying the progression of DN; such treatments are not a cure [3]. Hence, it highlights the need for novel therapeutic strategies for the DN.

Although persistent overdrive of the RAS resulting into the development of DN, the natriuretic peptide system (NPS) also get activated as a counter-regulatory circuit and postulated multitude of beneficial effects on renal vasculatures [4]. Consequently, neprilysin inhibitors (NEPi) which increase the bioavailability of natriuretic peptides are considered auxiliary to the RAS blockers in preventing chronic kidney diseases (CKD) [4–6]. Standalone NEPi and vasopeptidase inhibitors fails to make into the market due to unacceptably low efficacy and significant off-target effects (particularly angioedema), respectively, which prompted the development of new class of drug called as angiotensin receptor-neprilysin inhibitors (ARNi) [4–6]. LCZ696 (sacubitril/valsartan), the first member of ARNi class, convincingly proved advantageous over RAS blockers monotherapies in preventing CKD and cardiovascular diseases in preclinical and clinical studies [4–10]. Our recent report suggests that another such combination, thiorphan (NEPi)/telmisartan (ARB), abridged inflammation, fibrosis, and apoptosis thus prevented diabetic cardiomyopathy (DCM) [11]. However, the efficacy of thiorphan/telmisartan combination therapy yet to be tested against DN, therefore the present study aiming the same.

* Corresponding author at: Department of Pharmacy, Birla Institute of Technology and Science, Pilani, Pilani Campus, Pilani 333031, Rajasthan, India.
E-mail address: anil.gaikwad@pilani.bits-pilani.ac.in (A.B. Gaikwad).

https://doi.org/10.1016/j.lfs.2019.02.027
Received 3 February 2019; Received in revised form 10 February 2019; Accepted 11 February 2019
Available online 13 February 2019
0024-3205/

In contrast, angiotensin-converting enzyme 2 (ACE2), a monocarboxypeptidase of reno-protective and conventional RAS opposing depressor arm-angiotensin-(1–7) [Ang-(1–7)]/ACE2/MasR, is a potential therapeutic target to treat DN [1,12–14]. ACE2 is most abundantly expressed in kidney and heart with the highest affinity for angiotensin-II (Ang-II), cleaving its C-terminal amino acid and producing Ang-(1–7) [13]. Ang-(1–7) found superior to valsartan in preventing streptozotocin-induced DN by attenuating oxidative stress and inhibiting transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF) mediated pathways [15]. Our recent report demonstrated that an ACE2 activator diminazene aceturate (Dize) (15 mg/kg/day) treatment attenuated glomerular fibrosis and apoptosis via activation of protective Ang-(1–7)/ACE2/Ang-II type 2 receptor (AT2R) axis thus prevented DN [1]. Besides, the cyclic guanosine monophosphate (cGMP) mediated signallings are crucial for maintaining renal physiological functions, hence its dysfunction or deficit resulting in renal complications including the DN [16,17]. Notably, both the component of the combination therapy that we are targeting; NEPi and ACE2 activator could increase the levels of cGMP through two independent mechanisms. ACE2 activator will act on the soluble guanylyl cyclase (sGC) through Ang-(1–7)/ACE2/MasR or AT2R axis while NEPi will act on NPR-A/pGC-A by increasing the bioavailability natriuretic peptides, both resulting into increase the cGMP level [16–18]. Therefore, the present study conceived to test a hypothesis that, "the novel combination of NEPi and ACE2 activator possibly will protect the kidney against the development of DN".

2. Materials and methods

2.1. Materials

Supplementary Table 1.

2.2. Animals studies

All animal care and experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC), Birla Institute of Technology and Science Pilani (BITS-Pilani) under protocol No: IAEC/RES/20/07/Rev-1/22/11. Animal studies are reported following the ARRIVE guidelines [19]. Male Wistar rats (200 ± 10 g) were supplied by the Central Animal Facility of BITS-Pilani and were maintained under standard environmental conditions, with fed food and water ad libitum. We have injected a single dose of streptozotocin (55 mg/kg, i.p.) to induced diabetes in male Wistar rats [1,11,20]. Age-matched normal control (NC) rats received only vehicle (sodium citrate buffer). After 48 h of streptozotocin-injection, rats with plasma glucose levels > 16 mmol/L were considered as diabetic and included in the further studies.

2.3. Treatments regimens

Four weeks after the streptozotocin-injection, we randomly subdivided diabetic rats into six groups. i) DC-diabetic control, ii) DC + Th-thiorphan (0.1 mg/kg/day, p.o.), iii) DC + Tel-telmisartan (10 mg/kg/day, p.o.) and iv) DC + Dize-Dize (5 mg/kg/day, p.o.) monotherapies receiving DC, v) DC + Th/Tel-thiorphan/telmisartan, and vi) DC + Th/Dize-thiorphan/Dize combination therapies receiving DC [1,11]. All the treatments duration was four weeks. We kept eight rats in each group and maintained NC rats throughout the study ($n = 8$).

2.4. Plasma and urine biochemistry

At the end of the study, biochemical estimations for plasma glucose (PGL), albumin (PAL), creatinine (PCr), and blood urea nitrogen (BUN) were done using commercially available kits. Urine collection was done as per the protocol described previously and supernatant utilised for estimation of urine glucose (UGL), albumin (UAL) and creatinine (UCr) using commercially available kits [21]. Creatinine clearance (CrCL) was calculated by using formula; (CrCL) = [(UCr ∗ Urine volume)/PCr] and represented as ml/min per 100 g of body weight.

2.5. Assessment of RAS and NPS components levels by ELISA

We homogenised whole kidney in a recommended buffer solution, following by estimation of protein levels of ACE, ACE2, Ang-II, Ang-(1–7), neprilysin and cGMP using commercially available ELISA kits [1].

2.6. Picrosirius red staining

Picrosirius red (PSR) staining was performed as described previously [20]. Briefly, kidney sections of five-micro meter were cut, deparaffinized by using xylene, and stained with PSR. At least 25–30 kidney sections from each group were observed (4–5 section/kidney, total $n = 6$ kidney per group), and images of the glomerular and tubulointerstitial area were captured using an Olympus-BX41 microscope (Melville, NY, USA). Then, captured images were analysed semi-quantitative by using ImageJ software for measurement of % glomerular and tubulointerstitial fibrosis.

2.7. Immunoblotting

Protein isolation and immunoblotting were performed as per previously described protocols using rabbit/mouse monoclonal antibodies against p-NFκ-β(S-536) (#3033), IκBα (#4814), c-PARP (#5625), c-Caspase-3 (#9664), TGF-β (#3711), Smad7 (sc-11392) and β-actin (sc-4778) [Dilution 1:1000 (v/v)]. As secondary antibody, HRP conjugated anti-rabbit/mouse IgG was used at 1:20,000 (v/v) dilution. ECL system and Hyperfilm were used to detect proteins. Followed by densitometric analysis of immunoblots by using Image J software and obtained final data were analysed by using GraphPad Prism software (San Diego, CA, USA). We have expressed results as fold change over NC [1,11,20].

2.8. Statistical analysis

Statistical analysis of the data is complying with the recommendations on experimental design and analysis in pharmacology [22]. Experimental values are represented as Means ± S.E.M. (n = number of samples studied). One-way analysis of variance (ANOVA) was used for statistical comparison between different groups. If F value is significant, then Tukey test was applied for multiple comparisons. Data were considered as statistically significant if $p < 0.05$. GraphPad Prism, version 7.00 (San Diego, CA, USA) was used for data analysis.

3. Results

3.1. Telmisartan or Dize in combination with thiorphan prevented renal functional decline associated with DN

After eight weeks of streptozotocin-injection, levels of plasma glucose, creatinine and BUN were significantly higher, and plasma albumin level was significantly lower in DC rats in comparison to NC rats. Thiorphan (NEPi) and Dize (ACE2 activator) monotherapies had no effect on plasma glucose, whereas telmisartan (ARB) monotherapy and thiorphan/telmisartan (NEPi/ARB) and thiorphan/Dize (NEPi/ACE2 activator) combination therapies significantly reduced plasma glucose when compared to DC rats. Furthermore, telmisartan and Dize monotherapies, and both combination therapies abridged plasma creatinine and BUN levels and augmented plasma albumin levels when compared to DC rats. Interestingly, combination therapies significantly improved metabolic perturbations when compared to thiorphan monotherapy

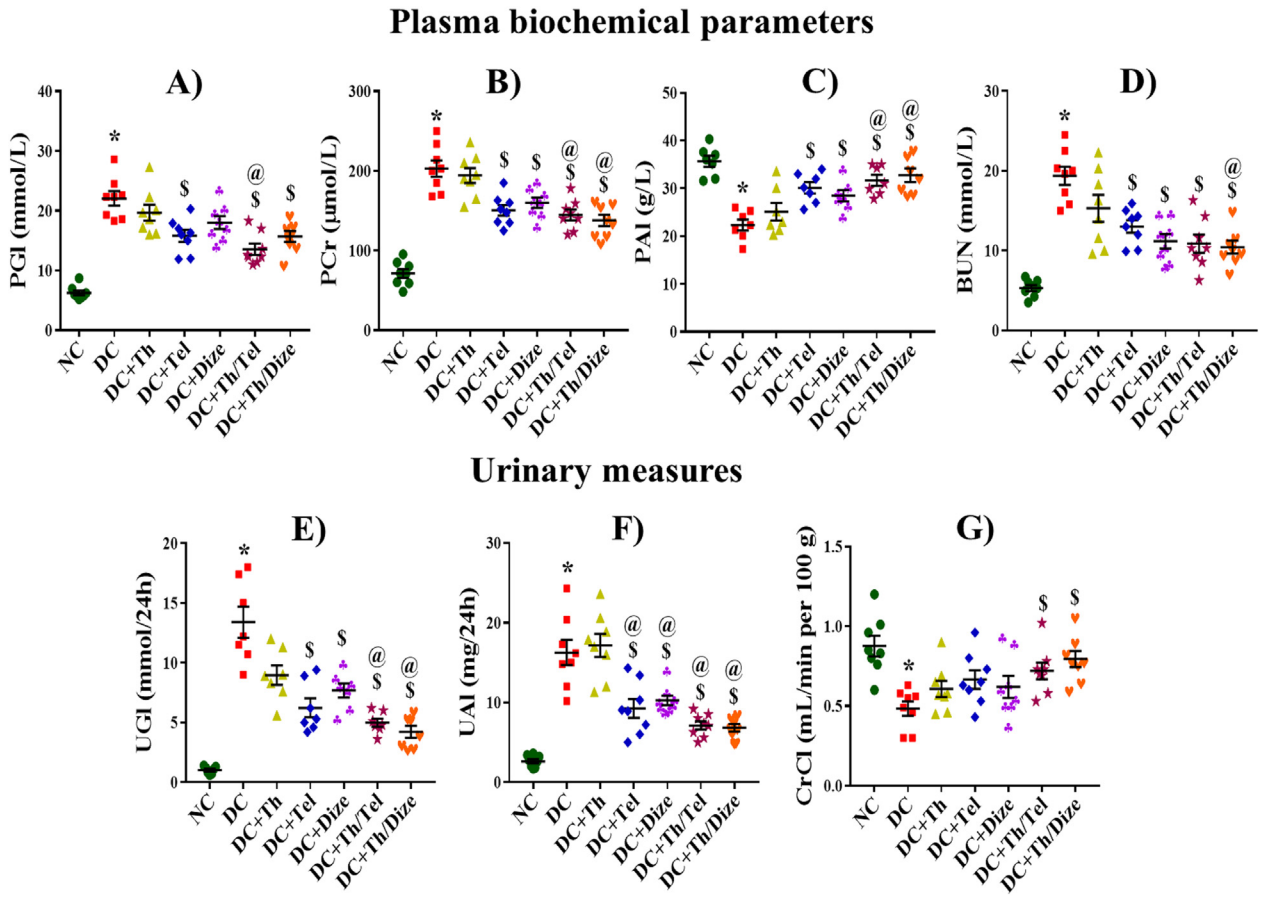

Fig. 1. Plasma and urine biochemistry.
Plasma and urine biochemistry were performed at the end of treatment period using commercially available spectrophotometric kits. Scattered plots, A)–D) displayed plasma levels of glucose (PGl), creatinine (PCr), albumin (PAl) and blood urea nitrogen (BUN), likewise, scattered plots, E)–G) represented urine levels of glucose (UGl), albumin (UCr) and urine creatinine clearances (CrCl), respectively. All the values are represented as mean ± SEM; $n = 8$. [(*) $p < 0.05$ vs NC; ($) $p < 0.05$ vs DC; (@) $p < 0.05$ vs DC + Th; (&) $p < 0.05$ vs DC + Dize].

also (Fig. 1A–D).

Moreover, urine biochemistry revealed that DC rats had increased urinary glucose, albumin and decreased creatinine clearance when compared to NC rats, which are clear indications of renal functional decline associated with DN. Thiorphan monotherapy did not affect urine biochemistry, whereas telmisartan and Dize monotherapies significantly reduced urinary glucose and albumin excretion with no change in creatinine clearance when compared to DC rats. Interestingly, both combination therapies significantly reduced urinary glucose and albumin excretion when compared to DC and thiorphan monotherapy receiving rats, and increased creatinine clearance when compared to DC rats (Fig. 1E–G).

3.2. Effects of thiorphan, telmisartan and Dize treatments on morphometric alterations associated with diabetes

DC rats displayed significantly decreased in body weight and increased kidney weight when compared to NC rats. Monotherapies did not affect body weight and kidney weight, while combination therapies only could reduce kidney weight significantly with no change in body weight when compared to DC rats. Relative kidney weight [(KW/BW) * 100] which serves an index of renal hypertrophy (a marker of DN), was increased in DC rats compared to NC rats [1,20]. Monotherapies showed no change in relative kidney weight, whereas combination therapies significantly reduced relative kidney weight when compared to DC rats (Table 1).

Table 1
Morphometric measures.
Morphometric parameters like body weight (BW), kidney weight (KW), and relative kidney weight (KW/BW * 100) were measured at the end of study.

	BW (g)	KW (g)	(KW/BW) * 100
NC	221 ± 18.6	1.12 ± 0.03	0.50 ± 0.02
DC	163 ± 12.8*	1.55 ± 0.09*	0.95 ± 0.07*
DC + Th	172 ± 15.0	1.52 ± 0.04	0.88 ± 0.06
DC + Tel	180 ± 12.2	1.42 ± 0.06	0.78 ± 0.04
DC + Dize	174 ± 7.45	1.40 ± 0.08	0.81 ± 0.06
DC + Th/Tel	191 ± 14.3	1.30 ± 0.05**	0.61 ± 0.04**
DC + Th/Dize	184 ± 10.1	1.25 ± 0.07**	0.66 ± 0.03**

Note: All the values are represented as mean ± SEM; $n = 8$.
* $p < 0.05$ vs NC.
** $p < 0.05$ vs DC.

3.3. Telmisartan or Dize in combination with thiorphan normalised renal RAS components levels in diabetic rats

In the kidney, DC rats showed a significant upsurge in ACE, ACE2, Ang-II, and reduction in Ang-(1–7) protein levels when compared to NC, resulting into significant augmentation in ACE/ACE2 and Ang-II/Ang-(1–7) ratio which has detrimental consequences on renal system (Fig. 2A–F). Thiorphan monotherapy exhibited no change in RAS components levels when compared with DC rats. Moreover, telmisartan monotherapy showed a reduction in ACE levels, ACE/ACE2 and Ang-II/Ang-(1–7) ratio with no change in ACE2, Ang-II and Ang-(1–7) levels,

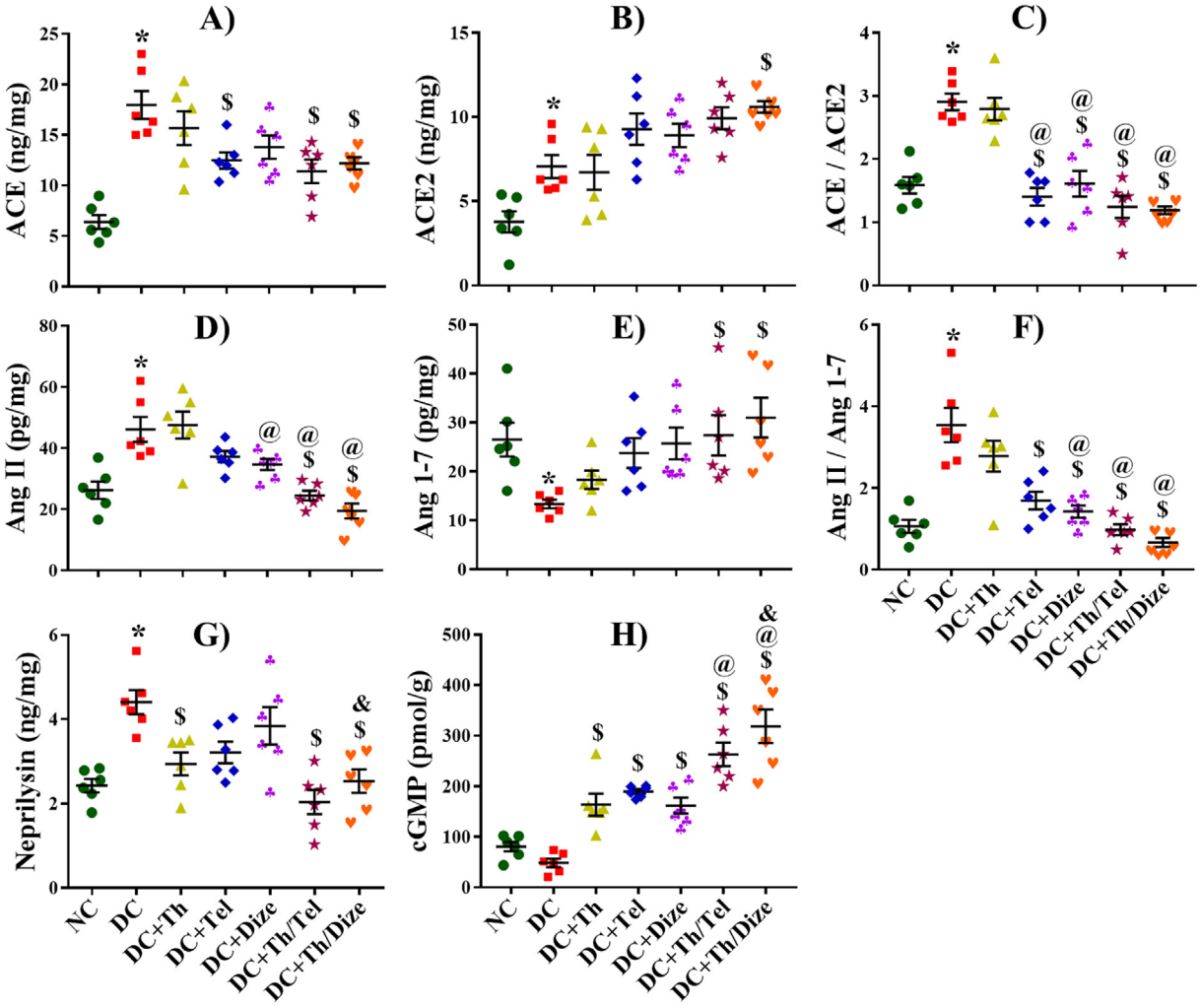

Fig. 2. Protein expressions of ACE, ACE2, Ang-II, Ang-(1–7), neprilysin and cGMP.
A) ACE, B) ACE2, C) Ang-II, D) Ang-(1–7), G) neprilysin and H) cGMP protein expressions in kidney were measured using commercially available ELISA kits. Similarly, scattered plots C) and F) represent ACE/ACE2 and Ang-II/Ang-(1–7) ratio in kidney, respectively. All the values are represented as mean ± SEM; $n = 6$. [(*) $p < 0.05$ vs NC; ($^{\$}$) $p < 0.05$ vs DC; ($^{@}$) $p < 0.05$ vs DC + Th; ($^{\&}$) $p < 0.05$ vs DC + Dize].

whereas Dize monotherapy demonstrated reduction in Ang-II levels, Ang-II/Ang-(1–7) and ACE/ACE2 ratio with no effect on ACE2, Ang-II, and Ang-(1–7) levels in kidney when compared with DC rats. In contrast, we have observed substantial normalisation of RAS component levels by combination therapies, evinced by a significant reduction in ACE and Ang-II levels, ACE/ACE2 ratio and Ang-II/Ang-(1–7), and increase in ACE2 (only by Th/Dize combination) and Ang-(1–7) levels when compared with DC rats. Interestingly, the reduction in Ang-II levels, ACE/ACE2, and Ang-II/Ang-(1–7) ratio was significant when compared with thiorphan monotherapy also (Fig. 2A–F).

3.4. Effects of thiorphan, telmisartan and Dize treatments on renal neprilysin and cGMP levels in diabetic rats

DC rats showed significantly increased neprilysin levels and no change in cGMP levels in the kidney when compared to NC rats. Renal neprilysin levels were not altered by telmisartan and Dize monotherapy whereas significantly reduced by thiorphan monotherapy and combination therapies when compared to DC rats. Th/Dize combination therapy reduced neprilysin levels in the kidney when compared to Dize monotherapy. Renal cGMP levels significantly increased by all the therapies when compared with DC rats. Interestingly, Th/Tel combination therapy showed increased cGMP levels when compared to thiorphan monotherapy, and Th/Dize combination treatment demonstrated increased cGMP levels when compared to thiorphan and Dize monotherapy (Fig. 2G–H).

3.5. Impact of thiorphan, telmisartan and Dize treatments on glomerular and tubulointerstitial fibrosis

PSR stained microscopical images analysis revealed that DC rats had substantial increased glomerular and tubulointerstitial fibrosis when compared to NC rats (Fig. 3). Thiorphan and Dize monotherapies did not affect renal fibrosis, while telmisartan monotherapy could reduce only glomerular fibrosis with no change in tubulointerstitial fibrosis when compared to DC rats. In contrast, combination therapies significantly abridged glomerular and tubulointerstitial fibrosis when compared to DC and thiorphan monotherapy receiving rats. In addition to this Th/Dize combination therapy significantly reduced tubulointerstitial fibrosis when compared to Dize monotherapy receiving rats (Fig. 3).

3.6. Telmisartan or Dize in combination with thiorphan prevented activation of NF-κB signalling in DN

In the kidney, DC rats exhibited increased p-NF-κB(S536)

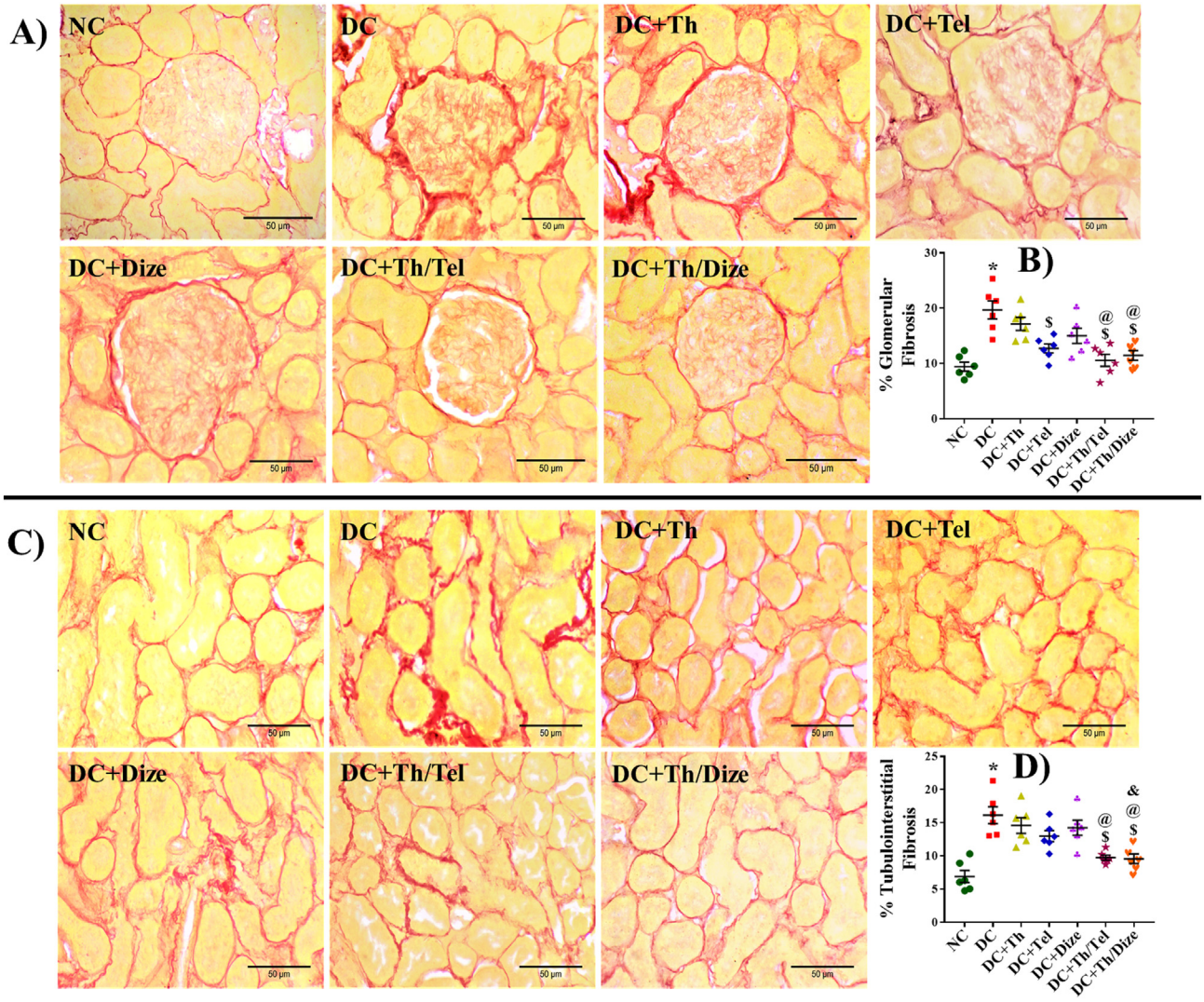

Fig. 3. Glomerular and tubulointerstitial fibrosis.
Representative microscopical images of PSR stained, kidney sections portraying A) glomerular and C) perivascular fibrosis (original magnification 1000× and Scale bar – 50 µm). Likewise, scattered plots B) and D) demonstrated percentage glomerular and perivascular fibrosis, respectively. All the values are represented as mean ± SEM; $n = 6$. [(*) $p < 0.05$ vs NC; ($) $p < 0.05$ vs DC; (@) $p < 0.05$ vs DC + Th; (&) $p < 0.05$ vs DC + Dize].

expressions and reduced IκBα (an inhibitory kinase for NF-κB activity) expressions when compared to NC rats, indicative of NF-κB signalling activation. Thiorphan monotherapy showed no change in p-NF-κB (S536) and IκBα expressions, whereas telmisartan and Dize monotherapies and combination therapies significantly reduced p-NF-κB (S536) and increased IκBα expressions when compared to DC. Interestingly, we observed reduction in p-NF-κB(S536) expressions by Th/Tel combination therapy in compared to thiorphan monotherapy and by Th/Dize combination therapy in compared to respective monotherapies (Figs. 4A–C and 5A–C).

3.7. Telmisartan or Dize in combination with thiorphan attenuated profibrotic TGF-β signalling in DN

DC rats showed increased TGF-β and reduced Smad7 (TGF-β negative regulator) expressions when compared to NC rats. Thiorphan monotherapy did not affect TGF-β and Smad7 expressions, while telmisartan monotherapy and Th/Tel combination therapy significantly reduced TGF-β expression when compared to DC rats and increased Smad7 expressions when compared to DC and thiorphan monotherapy (Fig. 4A, D–E). In contrast, Dize monotherapy could only increase Smad7 expression with no change in TGF-β expression when compared to DC, while Th/Dize combination significantly abridged TGF-β expression and elevated Samd7 expression when compared to DC and respective monotherapies (Fig. 5A, D–E).

3.8. Telmisartan or Dize in combination with thiorphan reduced apoptosis in DN

Expression of cell apoptosis markers, like c-PARP and c-Cas-3, were significantly increased in DC rats' kidney when compared to NC rats. Thiorphan monotherapy did not affect apoptosis markers expressions, whereas telmisartan, Dize monotherapies, and combination therapies reduced c-PARP and c-Cas-3 expressions when compared to DC (Figs. 4A, F–G, and 5A, F–G). Interestingly, Th/Tel combination therapy could only reduce c-PARP expression when compared to thiorphan monotherapy (Fig. 4A, F), while Th/Dize combination therapy reduced expression of both c-PARP and c-Cas-3 when compared to respective monotherapies (Fig. 5A, F–G).

4. Discussion

The prime aim of present investigation was to search a novel therapy for the treatment of DN, targeting the neprilysin inhibition and

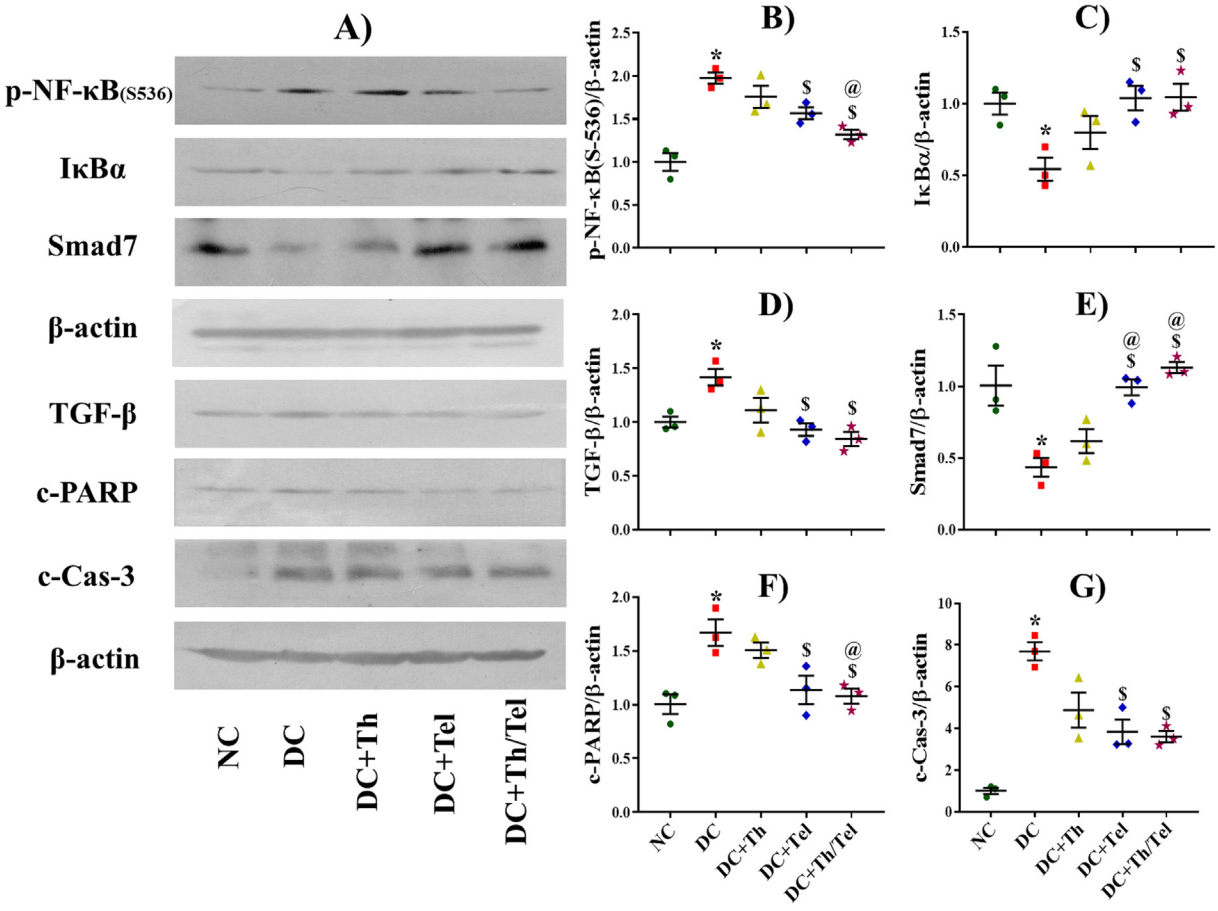

Fig. 4. Effect of Th/Tel combination therapy on protein expression of inflammatory, profibrotic and apoptotic markers.
A) Represented Western blot images and scattered plots B)–G) depicted fold change in protein expressions of p-NF-κB(S-536), IκBα, TGF-β, Smad7, c-PARP and c-Caspase-3 when compared with NC rats' kidney, respectively. β-actin was used as a loading control to normalised protein amount. All the values are represented as mean ± SEM; $n = 3$. [(*) $p < 0.05$ vs NC; ($) $p < 0.05$ vs DC; (@) $p < 0.05$ vs DC + Th].

RAS modulation, simultaneously. Given due consideration to existing literature and our previous reports, we have designed two combination therapies; i) thiorphan (NEPi)/telmisartan (ARB) and ii) thiorphan (NEPi)/Dize (ACE2 activator) to evaluate its efficacy against streptozotocin-induced DN in male Wistar rats. Diabetic rats exhibited substantial renal functional decline, as revealed by plasma and urine metabolic perturbations which were significantly prevented by four weeks of treatments with proposed combination therapies (Fig. 1). Additionally, the characteristic features of renal hypertrophy and DN such as increased actual and relative kidney weights were considerably attenuated by combination therapies (Table 1). As the combination therapies targeted both, NPS and RAS simultaneously, it normalised DN associated unfavourable changes in both the systems (Fig. 2). We observed elevated kidney cGMP levels in combination therapies receiving diabetic rats. Besides, histopathological evaluation by PSR staining confirmed augmented glomerular and tubulointerstitial fibrosis in the diabetic kidney which was significantly attenuated by combination therapies (Fig. 3). At molecular levels, combination therapy inhibited activation of inflammatory NF-κB signalling as evinced by decreased p-NF-κB(S536) and augmented IκBα levels in the kidney when compared to diabetic rats (Figs. 4–5). Likewise, combination treatments repressed profibrotic cascade by reducing TGF-β and increasing Smad7 expression in the kidney of diabetic rats (Figs. 4–5). The combination therapies also prevented diabetes-induced renal cell apoptosis as demonstrated by the significant reduction in c-PARP and c-Caspase-3 expression (Figs. 4–5). Hence, to the best of our knowledge, this is the first report, representing the renoprotective potential of a NEPi thiorphan in combination with either an ARB telmisartan or an ACE2 activator Dize against the development streptozotocin-induced DN in male Wistar rats.

Chronic hyperglycaemia plays a crucial pathogenic role in the development of DN. Hence, it should be of little surprise that therapeutic regimens ensuring good glycaemic control help in preventing or delaying the development of DN and the DCCT/EDIC study results confirmed the same [3,23]. Here, we observed that diabetic rats subjected to combination therapy showed a significant reduction in plasma glucose and urinary glucose excretion (Fig. 1). Clinically DN is characterised by albuminuria and glomerular hyperfiltration (reduced creatinine clearance) [3,23]. Here, we observed similar changes in diabetic rats which were significantly attenuated by combination therapies. Plasma biochemical measures governing kidney functions like PCr, PAL and BUN also normalised by combination therapies. Moreover, diabetic rats showed renal hypertrophy as indicated by increased actual and relative kidney weights, which were significantly reduced by combination therapy. In contrast, the body weight of diabetic rats remains unaltered by all the therapeutic regimens (Table 1).

The RAS itself has two counter regulatory arms, the pressor arm consists of Ang-II/ACE/AT1R axis, and the depressor arm comprises of Ang-(1–7)/ACE2/MasR or AT2R axis. In the development and progression of DN, detrimental role of the RAS is primarily attributed to the pressor arm, whereas the depressor arms components are reported for beneficial effects on the renal system [1,2,12–15]. Moreover, now it is established that autonomous paracrine and autocrine acting RASs exist in the kidney and its activation is key to the development of DN [24]. In the present study, diabetic rats exhibited an unfavourable alteration in intrarenal RAS (Fig. 2). Along with its AT1R antagonistic activity,

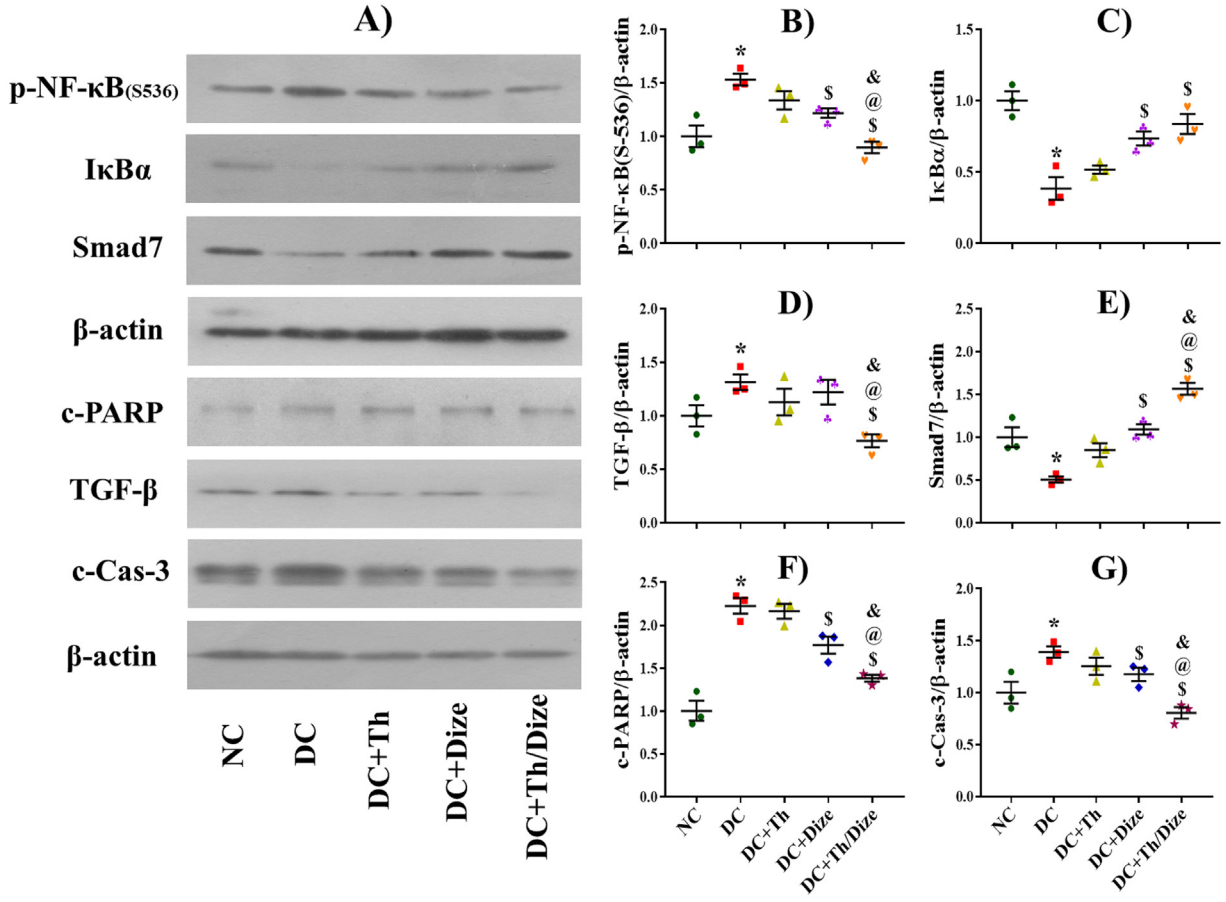

Fig. 5. Effect of Th/Dize combination therapy on protein expression of inflammatory, profibrotic and apoptotic markers.
A) Represented Western blot images and scattered plots B)–G) depicted fold change in protein expressions of p-NF-κB(S-536), IκBα, TGF-β, Smad7, c-PARP and c-Caspase-3 when compared with NC rats' kidney, respectively. β-actin was used as a loading control to normalised protein amount. All the values are represented as mean ± SEM; $n = 3$. [(*) $p < 0.05$ vs NC; ($) $p < 0.05$ vs DC; (@) $p < 0.05$ vs DC + Th; (&) $p < 0.05$ vs DC + Dize].

telmisartan reported to reduced Ang-II and increased ACE2 and MasR expressions in preventing diabetic renal complications [25,26]. In contrast, we observed that telmisartan monotherapy reduced ACE levels with no change in Ang-II, Ang-(1–7) and ACE2 levels in diabetic kidney. Previously, we reported that Dize (15 mg/kg/day) treatment decreased Ang-II and increased Ang-(1–7) and ACE2 levels in whole kidney and isolated glomeruli of diabetic rats [1]. Since we have used lower dose (5 mg/kg/day) of Dize in the present study, its monotherapy could reduce Ang-II levels only with no change in ACE, ACE2 and Ang-(1–7) levels in diabetic kidney. Although thiorphan monotherapy did not affect RAS components levels, its combination therapy with either telmisartan or Dize significantly reduced ACE and Ang-II levels and increased ACE2 (by Th/Dize combination only) and Ang-(1–7) levels in preventing DN (Fig. 2A–B and D–E). Augmented ACE/ACE2 and Ang-II/Ang-(1–7) ratio reported to damage normal renal physiology [1,27]. Here, we observed increased renal ACE/ACE2 and Ang-II/Ang-(1–7) ratio in diabetic rats, which were significantly attenuated by telmisartan and Dize monotherapy and both combination therapies (Fig. 2C and F).

On the other hand, the conventional RAS opposing natriuretic peptides act through the NPR-A/pGC-A pathway and induces vasodilation, natriuresis, and diuresis, thereby helps in maintaining blood pressure, fluid and electrolytes balance. Neprilysin, a zinc-dependent type II integral membrane metallopeptidase catabolized natriuretic peptides thus clear them from systemic circulation [4]. Diabetic individuals showed increased neprilysin level in urine, and neprilysin inhibition on the top of ARB delayed the development of DN, clinically [28,29]. In our study, diabetic rats showed augmented neprilysin levels in the kidney which were significantly attenuated by thiorphan monotherapy and its combination therapy with telmisartan or Dize (Fig. 2G). Besides, faulty NPR-A/pGC-A/cGMP/PKG and NO/sGC/cGMP signalling involved in the renal dysfunctions.

Consequently, agents increasing cGMP levels by acting on either of these pathways (e.g., phosphodiesterase-5 inhibitors, NEPi, recombinant natriuretic peptides, and sGC stimulator) found beneficial in preventing renal complications including DN [16–18,30]. Here, we have observed that thiorphan, telmisartan and Dize monotherapies, and both combination therapies increased renal cGMP levels when compared to diabetic rats. However, the increase was more pronounced in rats receiving combination therapies.

Next, to explore the underlying molecular mechanisms responsible for the renoprotective potential of proposed combination therapies, we have checked the expressions of important molecules of inflammatory, profibrotic and apoptotic pathways. Hyperglycaemia driven activation of these pathways contributes to the pathogenesis of DN hence its suppression is a good tactic to prevent the development of DN [1,31–33]. In diabetic condition, NF-κB signalling gets activated by dissociation of inactive NF-κB/IκBα complex and subsequent nuclear translocation of transcription factor phosphorylated NF-κB, ultimately resulting into increased expressions of proinflammatory cytokines, adhesion molecules and angiogenic mediators [32]. In this regard, we observed augmented p-NF-κB(S536) and abridged IκBα expression in diabetic rats' kidney indicating activation of the NF-κB signalling. In CKD induced by 5/6 nephrectomy in male Sprague-Dawley rats, LCZ696 treatment inhibited NF-κB activation and reduced expressions of its downstream molecules like MCP-1, NADP oxidase-4,

myeloperoxidase, and cyclooxygenase-2, consequently limiting cardiovascular and renal functional decline [7,9]. Since we are the first exploring NEPi and ACE2 activator combination against DN, no reports are available on thiorphan/Dize combination's effects on the pathological signalling. Rajapaksha et al. reported that Dize exposure to lipopolysaccharide-stimulated Kupffer cells inhibited NF-kB activity, reduced MCP-1 and IL-6 gene expressions [34]. In the present study, telmisartan and Dize monotherapy and combination therapies showed significant inhibition of NF-κB signalling in diabetic rats' kidney (Figs. 4–5).

In progressive DN, profibrotic TGF-β signalling considered as the 'master regulator'. It drives extracellular matrix accumulation, glomerular and tubulointerstitial fibrosis and subsequent normal tissue structure damage, resulting in end-stage renal disease [20,31]. We observed increased TGF-β and decreased Smad7 (negative regulator of TGF-β) expressions and resulting augmented glomerular and tubulointerstitial fibrosis in diabetic rats' kidney. Recently, we have demonstrated inhibition of TGF-β signalling and myocardial fibrosis by thiorphan/telmisartan combination in preventing DCM [11]. ACE2 activation by Dize (15 mg/kg/day) reduced TGF-β and fibrotic marker collagen IV and fibronectin expressions in glomeruli of streptozotocin-induced diabetic male Wistar rats [1]. Interestingly, in the present study combination therapies inhibited TGF-β signalling and attenuated glomerular and tubulointerstitial fibrosis thus halted DN progression (Figs. 3–5).

Furthermore, diabetic rats' kidney exhibited substantial apoptosis evinced by increased expressions of c-PARP and c-Caspase when compared normal control rats. Our previous studies unveiled that thiorphan/telmisartan combination therapy and Dize monotherapy reduced apoptosis in heart and kidney of diabetic rats, thereby prevented the development of DCM and DN, respectively [1,11]. Similarly, in the present study, we have observed markedly reduced apoptosis in the kidney of rats receiving telmisartan and Dize monotherapy and thiorphan/telmisartan and thiorphan/Dize combination therapy (Figs. 4–5). One of the notable findings of the present study is that the renoprotection against DN achieved by proposed combination therapies of NEPi/ARB (ARNi) and NEPi/ACE2 activator was superior than the respective monotherapies in all the aspect. Nevertheless, we have not observed statistically significant deference in any of the measured parameters among both combination therapies. If we take degree of improvement into the consideration than, Th/Dize combination was better over Th/Tel combination in attenuating renal functional decline and pathological signalling cascades.

5. Conclusion

Streptozotocin-induced diabetic male Wistar rats when subjected to NEPi/ARB (ARNi) and NEPi/ACE2 activator combination therapies demonstrated alleviated metabolic alteration, improved renal functions, normalised RAS and NPS components, reduced glomerular and tubulointerstitial fibrosis and suppressed inflammatory, profibrotic and apoptotic signalling, ultimately prevented the development of DN. Hence, we can say that NEPi/ARB and NEPi/ACE2 activator combination therapies might be novel therapies in preventing the development of DN and other diabetic complications.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.02.027.

Acknowledgement

A.B.G. acknowledges the Science and Engineering Research Board-Department of Science & Technology (SERB-DST), Govt. of India [SERB/ECR/2017/000317] for the financial support for this work. V.M. sincerely acknowledges the Indian Council of Medical Research (ICMR) for senior research fellowship [45/10/2018-PHA/BMS/OL].

Conflict of interest

None declared.

References

[1] S.K. Goru, A. Kadakol, V. Malek, A. Pandey, N. Sharma, A.B. Gaikwad, Diminazene aceturate prevents nephropathy by increasing glomerular Ace2 and At2 receptor expression in a rat model of type1 diabetes, Br. J. Pharmacol. 174 (2017) 3118–3130.
[2] T. Micakovic, S. Papagiannarou, E. Clark, Y. Kuzay, K. Abramovic, J. Peters, C. Sticht, N. Volk, T. Fleming, P. Nawroth, The angiotensin II type 2 receptors protect renal tubule mitochondria in early stages of diabetes mellitus, Kidney Int. 94 (2018) 937–950.
[3] H. Gallagher, R. Suckling, Diabetic nephropathy: where are we on the journey from pathophysiology to treatment? Diabetes Obes. Metab. 18 (2016) 641–647.
[4] V. Malek, A.B. Gaikwad, Neprilysin inhibitors: a new hope to halt the diabetic cardiovascular and renal complications? Biomed. Pharmacother. 90 (2017) 752–759.
[5] P. Judge, R. Haynes, M.J. Landray, C. Baigent, Neprilysin inhibition in chronic kidney disease, Nephrol. Dial. Transplant. 30 (2015) 738–743.
[6] K.F. Docherty, J.J. McMurray, Angiotensin receptor-neprilysin inhibitors: a new paradigm in heart failure with reduced ejection fraction, Int. J. Cardiol. (2018), https://doi.org/10.1016/j.ijcard.2018.1005.1124.
[7] Y. Suematsu, W. Jing, A. Nunes, M.L. Kashyap, M. Khazaeli, N.D. Vaziri, H. Moradi, LCZ696 (sacubitril/valsartan), an angiotensin-receptor neprilysin inhibitor, attenuates cardiac hypertrophy, fibrosis and vasculopathy in a rat model of chronic kidney disease, J. Card. Fail. 24 (2018) 266–275.
[8] R. Haynes, P.K. Judge, N. Staplin, W.G. Herrington, B.C. Storey, A. Bethel, L. Bowman, N. Brunskill, P. Cockwell, M. Hill, Effects of sacubitril/valsartan versus irbesartan in patients with chronic kidney disease: a randomised double-blind trial, Circulation 38 (2018) 1505–1514.
[9] W. Jing, N.D. Vaziri, A. Nunes, Y. Suematsu, T. Farzaneh, M. Khazaeli, H. Moradi, LCZ696 (sacubitril/valsartan) ameliorates oxidative stress, inflammation, fibrosis and improves renal function beyond angiotensin receptor blockade in CKD, Am. J. Transl. Res. 9 (2017) 5473–5484.
[10] P.M. Nielsen, D. Grimm, M. Wehland, U. Simonsen, M. Krüger, The combination of valsartan and sacubitril in the treatment of hypertension and heart failure–an update, Basic Clin. Pharmacol. Toxicol. 122 (2018) 9–18.
[11] V. Malek, A.B. Gaikwad, Telmisartan and thiorphan combination treatment attenuates fibrosis and apoptosis in preventing diabetic cardiomyopathy, Cardiovasc. Res. 115 (2019) 373–384.
[12] R.A.S. Santos, W.O. Sampaio, A.C. Alzamora, D. Motta-Santos, N. Alenina, M. Bader, M.J. Campagnole-Santos, The ACE2/angiotensin-(1–7)/MAS axis of the renin-angiotensin system: focus on angiotensin-(1–7), Physiol. Rev. 98 (2017) 505–553.
[13] M.J. Ross, M. Nangaku, ACE2 as therapy for glomerular disease: the devil is in the detail, Kidney Int. 91 (2017) 1269–1271.
[14] D. Batlle, J. Wysocki, M.J. Soler, K. Ranganath, Angiotensin-converting enzyme 2: enhancing the degradation of angiotensin II as a potential therapy for diabetic nephropathy, Kidney Int. 81 (2012) 520–528.
[15] K. Zhang, X. Meng, D. Li, J. Yang, J. Kong, P. Hao, T. Guo, M. Zhang, Y. Zhang, C. Zhang, Angiotensin (1–7) attenuates the progression of streptozotocin-induced diabetic renal injury better than angiotensin receptor blockade, Kidney Int. 87 (2015) 359–369.
[16] Y. Chen, J.C. Burnett, Particulate guanylyl cyclase A/cGMP signaling pathway in the kidney: physiologic and therapeutic indications, Int. J. Mol. Sci. 19 (2018) 1006–1018.
[17] S.M. Krishnan, J.R. Kraehling, F. Eitner, A. Bénardeau, P. Sandner, The impact of the nitric oxide (NO)/soluble guanylyl cyclase (sGC) signaling cascade on kidney health and disease: a preclinical perspective, Int. J. Mol. Sci. 19 (2018) 1712–1730.
[18] A. Buglioni, J.C. Burnett Jr., New pharmacological strategies to increase cGMP, Annu. Rev. Med. 67 (2016) 229–243.
[19] C. Kilkenny, W.J. Browne, I.C. Cuthill, M. Emerson, D.G. Altman, Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research, PLoS Biol. 8 (2010) e1000412.
[20] S.K. Goru, A. Kadakol, A. Pandey, V. Malek, N. Sharma, A.B. Gaikwad, Histone H2AK119 and H2BK120 mono-ubiquitination modulate SET7/9 and SUV39H1 in type 1 diabetes-induced renal fibrosis, Biochem. J. 473 (2016) 3937–3949.
[21] V. Malek, N. Sharma, A.B. Gaikwad, Histone acetylation regulates natriuretic peptides and neprilysin gene expressions in diabetic cardiomyopathy and nephropathy, Curr. Mol. Pharmacol. 12 (2019) 61–71.
[22] M.J. Curtis, R.A. Bond, D. Spina, A. Ahluwalia, S. Alexander, M.A. Giembycz, A. Gilchrist, D. Hoyer, P.A. Insel, A.A. Izzo, Experimental design and analysis and their reporting: new guidance for publication in BJP, Br. J. Pharmacol. 172 (2015) 3461–3471.
[23] I.H. De Boer, D.E.r. group, Kidney disease and related findings in the diabetes control and complications trial/epidemiology of diabetes interventions and complications study, Diabetes Care 37 (2014) 24–30.
[24] R.M. Carey, H.M. Siragy, The intrarenal renin–angiotensin system and diabetic nephropathy, Trends Endocrinol. Metab. 14 (2003) 274–281.
[25] A.P. Lakshmanan, K. Watanabe, R.A. Thandavarayan, F.R. Sari, M. Harima, V.V. Giridharan, V. Soetikno, M. Kodama, Y. Aizawa, Telmisartan attenuates oxidative stress and renal fibrosis in streptozotocin induced diabetic mice with the

alteration of angiotensin-(1–7) mas receptor expression associated with its PPAR-γ agonist action, Free Radic. Res. 45 (2011) 575–584.
[26] A. Nishiyama, T. Nakagawa, H. Kobori, Y. Nagai, N. Okada, Y. Konishi, T. Morikawa, M. Okumura, I. Meda, H. Kiyomoto, Strict angiotensin blockade prevents the augmentation of intrarenal angiotensin II and podocyte abnormalities in type 2 diabetic rats with microalbuminuria, J. Hypertens. 26 (2008) 1849.
[27] S. Bernardi, B. Toffoli, C. Zennaro, C. Tikellis, S. Monticone, P. Losurdo, G. Bellini, M.C. Thomas, F. Fallo, F. Veglio, High-salt diet increases glomerular ACE/ACE2 ratio leading to oxidative stress and kidney damage, Nephrol. Dial. Transplant. 27 (2011) 1793–1800.
[28] M. Packer, B. Claggett, M.P. Lefkowitz, J.J. McMurray, J.L. Rouleau, S.D. Solomon, M.R. Zile, Effect of neprilysin inhibition on renal function in patients with type 2 diabetes and chronic heart failure who are receiving target doses of inhibitors of the renin-angiotensin system: a secondary analysis of the PARADIGM-HF trial, Lancet Diabetes Endocrinol. 6 (2018) 547–554.
[29] S. Gutta, N. Grobe, M. Kumbaji, H. Osman, M. Saklayen, G. Li, K.M. Elased, Increased urinary angiotensin converting enzyme 2 (ACE2) and neprilysin (NEP) in type 2 diabetic patients, Am. J. Physiol. Ren. Physiol. 315 (2018) F263–F274.
[30] N.M. Idzerda, M.J. Pena, D. de Zeeuw, H.J. Heerspink, Future and Novel Compounds in the Treatment of Diabetic Nephropathy, Diabetic Nephropathy, Springer, 2019, pp. 515–539.
[31] A.S. Chang, C.K. Hathaway, O. Smithies, M. Kakoki, Transforming growth factor β1 and diabetic nephropathy, Am. J. Physiol. Ren. Physiol. 310 (2016) F689–F696.
[32] J. Wada, H. Makino, Inflammation and the pathogenesis of diabetic nephropathy, Clin. Sci. 124 (2013) 139–152.
[33] C.M.O. Volpe, P.H. Villar-Delfino, P.M.F. Anjos, J.A. Nogueira-Machado, Cellular death, reactive oxygen species (ROS) and diabetic complications, Cell Death Dis. 9 (2018) 119–128.
[34] I.G. Rajapaksha, K.Y. Mak, P. Huang, L.M. Burrell, P.W. Angus, C.B. Herath, The small molecule drug diminazene aceturate inhibits liver injury and biliary fibrosis in mice, Sci. Rep. 8 (2018), https://doi.org/10.1038/s41598-41018-28490-y.