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Original article

Simultaneous inhibition of neprilysin and activation of ACE2 prevented diabetic cardiomyopathy

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ABSTRACT

Background: Neprilysin inhibitors (NEPi) are assisting the renin-angiotensin system (RAS) inhibitors in halting diabetic cardiomyopathy (DCM). Away from conventional tactic, a recent report revealed the renoprotective potential of NEPi and angiotensin-converting enzyme (ACE2) activator combination therapy against diabetic nephropathy. However, this combination so far not evaluated against DCM, thus the present investigation aiming the same.

Methods: Streptozotocin-induced (55 mg/kg, *ip*) type 1 diabetic (T1D) male Wistar rats were treated with either monotherapy of thiorphan (0.1 mg/kg/day, *po*) or diminazene aceturate (5 mg/kg/day, *po*), or their combination therapy, for four weeks. After hemodynamic measurements, all the rats' heart and plasma were collected for biochemistry, ELISA, histopathology, and immunoblotting.

Results: Metabolic perturbations and failing cardiac functions associated with diabetes were markedly attenuated by combination therapy. Besides, unfavourable alterations in RAS and natriuretic peptides system (NPS) were corrected by combination therapy. Interestingly, combination therapy significantly increased plasma and heart cGMP levels compared to T1D and monotherapy receiving rats. Moreover, rats receiving combination therapy exhibited significant inhibition of activated NF-κB, TGF-β and apoptotic signalling, and a notable reduction in cardiac fibrosis when compared to T1D rats. Expressions of posttranslational histone modifications markers; H3K4Me2 and its methyltransferases (SET7/9 and RBBP5) were significantly enhanced in T1D hearts, which were significantly reduced by combination therapy.

Conclusions: The NEPi and ACE2 activator combination therapy effectively prevented DCM by normalising RAS and NPS activities, increasing cGMP, inhibiting inflammatory, pro-fibrotic and apoptotic signalling, and reversing H3K4Me2 and its methyl transferases expressions.

Introduction

Hyperglycaemia derived alterations in two counter-regulatory constraints, the renin-angiotensin system (RAS) and natriuretic peptide system (NPS) are the main culprits for the development and progression of diabetic cardiomyopathy (DCM) [1]. The neprilysin inhibitor (NEPi) potentially improves the protective effects of RAS blockade (either by angiotensin receptor blocker (ARB) or *ACE* inhibitor (ACEi)) against cardiovascular diseases (CVD) by increasing the levels of the natriuretic peptides, although there is an insignificant clinical benefit of NEPi monotherapy [1]. The first ever angiotensin receptor-neprilysin inhibitors (ARNi), LCZ696 (sacubitril/valsartan), proved its primacy over ARB or ACEi monotherapies in preventing hypertension, cardiomyopathy and heart failure in preclinical and clinical studies [1–7]. We have reported that another such combination of telmisartan and thiorphan prevented DCM by inhibiting inflammatory, profibrotic and apoptotic signalling in streptozotocin-induced type 1 diabetic (T1D) male Wistar rats [8]. However, in search of the better alternative regimen, away from the conventional strategies, the present study aimed at evaluating the efficacy of ACE2 activator and NEPi combination treatment against DCM.

The RAS is composed of two arms, the pressor arm, also known as the conventional RAS (Ang-II/ACE/AT1R), and the depressor arm, also called as the non-conventional RAS (Ang-(1-7)/ACE2/MasR). Both arms of RAS opposed each other activities, thereby regulates vasoconstriction and vasodilation and assist in maintaining hemodynamic and other homeostasis [9,10]. Till now research has mainly focused on inhibition of the pressor arm, however right side of devil RAS, activation of the depressor arm is less explored in

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DCM. The monocarboxypeptidase ACE2 is a prime therapeutic target from the depressor arm. ACE2 act by removing single amino acids from the C-terminus of Ang-I and Ang-II thus produced Ang-(1–9) and Ang-(1–7), respectively. These resulting peptides reported having a protective role against metabolic disorders, hypertension and heart failure [9–11]. An ACE2 activator diminazene aceturate (Dize) significantly attenuated the myocardial infarction induced a decrease in fractional shortening, improved max-*dp/dt* and prevented ventricular hypertrophy [12]. Dize treatment reversed electrical deviations in ventricular repolarisation and arrhythmic cardiac markers in streptozotocin-induced T1D male Wistar rats [13]. Interestingly, cyclic guanosine monophosphate (cGMP) is well reported for its beneficial effects on the cardiovascular system [14]. ACE2 activator can increase the level of cGMP *via* activation of the PI3K/Akt pathway through Ang-(1–7)/ACE2/MasR or AT2R axis [9–11]. Likewise, NEPi also can do the same by increasing the bioavailability of the natriuretic peptide [1]. In these regards, our recent report suggests that in diabetic kidney NEPi (thiorphan) and ACE2 activator (Dize) combination therapy normalised RAS components, increased cGMP levels, inhibited inflammation, fibrosis, and apoptosis consequently prevented diabetic nephropathy (DN) [15]. Nevertheless, combination therapy of thiorphan/Dize is so far not explored against DCM. Hence, different from common tactic, in the present study we aimed to evaluate cardioprotective potential NEPi and ACE2 activator combination therapy against DCM and to explore the molecular mechanisms responsible for the same.

Materials and methods

Chemicals

Streptozotocin and diminazene aceturate (Dize) were purchased from Sigma (St. Louis, MO, USA). Thiorphan was purchased from Cayman Chemical (Ann Arbor, MI, USA). Biochemical parameter estimations kits (glucose, creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH)) were purchased from Accurex Biomedical Pvt. Ltd. (Mumbai, Maharashtra, India). ANP, BNP, and neprilysin ELISA kits were purchased from Ray Biotech (Norcross, GA, USA), cGMP and Ang-II ELISA kits were purchased from Elabscience (Wuhan, China), while ACE2, ACE, and Ang-(1–7) ELISA kits were purchased from Wuhan Fine Biological Technology (Wuhan, China). Anti-Smad7 and anti-β-actin primary antibodies were procured from Santa-Cruz Biotechnology (Dallas, Texas, USA) and all other primaries and secondaries antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). All the other chemicals were purchased from Sigma unless otherwise mentioned.

Animal studies and drug treatments

All the animal experiment protocols were approved by the Institutional Animal Ethics Committee, Birla Institute of Technology and Science Pilani (BITS-Pilani) (Protocol Approval No: IAEC/RES/20/07/Rev-1/22/11). We have reported animal study conferring the ARRIVE guidelines [16]. Adult male Wistar rats (200 ± 10 gm) were supplied by the Central Animal Facility (CAF) BITS-Pilani and were kept under standard environmental conditions, with food and water *ad libitum*. As described previously, a single dose of streptozotocin (55 mg/kg, *ip*) dissolved in ice-cold sodium citrate buffer (0.01 M, pH 4.4) was used to induce type 1 diabetes and after 48 h of streptozotocin-injection rats showing plasma glucose levels >16 mmol/l were considered type 1 diabetic (T1D) and included in further study (n = 32) [8,9,17]. After four weeks of diabetes induction, initial plasma biochemical assessments were done, and T1D rats were subdivided into four group i) T1D rats, ii) T1D rats receiving thiorphan monotherapy (0.1 mg/kg/day, *po*)-T1D + Th [8], iii) T1D rats receiving Dize monotherapy (5 mg/kg/day, *po*)- T1D + Dize, and iv) T1D rats subjected to thiorphan and Dize combination therapy- T1D + Com [15]. All the treatments duration was four weeks and age match nondiabetic (ND) rats were maintained throughout the study. We have kept eight rats in each experimental group (n = 8 rats/group).

Plasma biochemistry

At the end of the study, blood samples were collected, and plasma samples were assayed for glucose (PGL), lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) using commercially available spectrophotometric kits.

Haemodynamic measurements

Systemic blood pressure and left ventricular (LV) functional measures were evaluated by using the PowerLab system (AD Instruments, Bella Vista, NSW, Australia) as described previously [8]. In brief, the rats were anaesthetised with sodium pentobarbital (Nembutal®, 50 mg/kg, *ip*) and the right carotid artery cannulation was done by inserting a polyethylene cannula (PE-50, Clay Adams). This carotid cannula was connected to a liquid pressure transducer (MLT844) interfaced to PowerLab signal transduction unit. After a half an hour of stabilisation, blood pressure (BP) was recorded for the next half an hour. For the evaluation of LV functional parameters, the carotid cannula was advanced cautiously into left vertical until we observed maximum systolic BP (SBP) and diastolic BP (DBP) near to zero. After a ten-minute stabilisation, the LV functional parameters were recorded for the next half an hour. Data analysis was done by using Lab Chart-8 program supplied with the PowerLab system. After completion of IBP measurements, animals were euthanized by an overdose of anaesthesia (Nembutal®, 120 mg/kg, *ip*) and hearts were collected for further experiments.

Assessment of RAS and NPS components levels in plasma and heart by ELISA

Heart tissues were homogenised, and plasma samples were diluted in the recommended buffer solution, then protein levels of Ang-II, Ang-(1–7), ACE, ACE2, neprilysin, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and cGMP were measured by using commercially available ELISA kits following the manufacturer's instructions.

Histopathological evaluation of cardiac fibrosis by picrosirius red staining

Cardiac fibrosis was examined by picrosirius Red (PSR) staining as described previously [8]. Briefly, heart tissues were fixed in 10% formalin, embedded in paraffin blocks, sectioned (5 μm) by microtome, followed by PSR staining. From each group at least six different hearts microscopical slides were observed and imaged using an Olympus-BX41 microscope (Melville, NY, USA). Images were analysed semi-quantitatively by using ImageJ software to measure % PSR positive area (collagen deposition).

Immunoblotting

Protein isolation and western blotting were done as previously described protocols [8,9], using mouse/rabbit monoclonal antibodies against: p-NFκ-β(S-536) (#3033), p-IκBα (S-32) (#2859), c-PARP (#5625), c-Caspase-3 (#9664), TGF-β

(#3711), Smad7 (sc-11392), SET7/9 (#2813), SET8 (#2996), RBBP5 (#13171), H3K4Me2 (#9725), histone 3 (H3) (#4499) and β-actin (sc-4778) [dilution 1:1000 (v/v)]. As secondary antibody, we utilised HRP conjugated anti-mouse/rabbit IgG at 1:20000 (v/v) dilution. Proteins were visualised by ECL system and Hyperfilm, followed by densitometric quantification using Image J software. All exposures were in a linear dynamic range. β-actin and H3 expressions were used as loading controls, and results were expressed as fold changes over ND.

Statistical analysis

We represented experimental values as means ± SD where 'n' refers to a number of samples studied. Statistical comparison between different groups was made using one-way ANOVA, followed by multiple comparisons of two groups mean by Tukey test (if F value is significant). We used GraphPad Prism-7 software (San Diego, CA, USA) for statistical analysis and considered data as statistically significantly different if $p < 0.05$.

Results

Thiorphan and Dize combination therapy prevented diabetes-associated metabolic and morphometric perturbations

T1D rats showed significant hyperglycaemia and, increased CK-MB and LDH (molecular markers of cardiomyopathy) levels when compared to ND rats. Thiorphan and Dize monotherapies did not change these metabolic measures when compared to T1D rats. Interestingly, combination therapy displayed a marked reduction in hyperglycaemia, CK-MB and LDH levels when compared to T1D and thiorphan monotherapy receiving rats (Table 1). T1D rats exhibited a marked reduction in body weight (BW) and heart weight (HW) comparative to ND rats. HW and BW remained unchanged across all three treatment groups when compared to T1D rats. Moreover, HW/BW ratio was markedly increased in T1D rats when compared to ND rats. Thiorphan or Dize monotherapy did not change the HW/BW ratio, while their combination therapy significantly reduced the HW/BW ratio when compared to T1D rats (Table 1).

Thiorphan and Dize combination therapy attenuated the systemic haemodynamic and left ventricular functional decline in T1D rats

Before starting treatments (four weeks after streptozotocin-injection), in comparison to ND rats, T1D rats had increased systolic blood pressure (SBP) (108.9 ± 3.26 *vs.* 139.61 ± 8.90 mmHg, $p < 0.05$), diastolic blood pressure (DBP) (82.1 ± 2.21 *vs.* 93.2 ± 1.89 mmHg, $p < 0.05$), mean arterial pressure (MAP) (90.02 ± 2.07 *vs.* 108.89 ± 2.56 mmHg, $p < 0.05$), LV-systolic pressure (LVSP) (111.3 ± 5.90 *vs.* 140.0 ± 12.1 mmHg, $p < 0.05$) and LV-end diastolic pressure (LVEDP) (2.20 ± 0.20 *vs.* 10.2 ± 0.71 mmHg, $p < 0.05$). In contrast, eight weeks after streptozotocin-injection (at the end of study), T1D rats displayed reduced SBP, DBP, MAP, heart rate (HR), LVSP, Max-*dp/dt* and Min-*dp/dt*, and increased LVEDP when compared to ND rats. HR was increased, whereas SBP, DBP, and MAP did not change in thiorphan or Dize monotherapy receiving rats when compared to T1D rats (Table 1). Thiorphan or Dize monotherapy and their combination therapy could increase LVSP, Max-*dp/dt*, Min-*dp/dt* and decrease LVEDP when compared to T1D rats (Table 1). However, the degree of improvement in these indices was better in rats subjected to combination therapy than respective monotherapies.

Combination therapy of thiorphan and Dize normalised systemic and local RAS components in T1D rats

T1D rats demonstrated significantly increased ACE, Ang-II, and reduced Ang-(1–7) levels in plasma when compared to ND. In contrast, systemic ACE2 levels remain unchanged across the groups. Dize monotherapy and combination therapy significantly reduced ACE, Ang-II, and increased And-(1–7) levels in plasma when compared to T1D rats. Interestingly, systemic Ang-II levels in both Dize monotherapy and combination therapy, and ACE levels in combination therapy were significantly lowered when compared to thiorphan monotherapy (Fig. 1A–D). Likewise, T1D rats' hearts exhibited significantly augmented ACE, Ang-II and abridged ACE2, Ang-(1–7) levels when compared to ND rats. Thiorphan monotherapy did no change RAS components levels when compared to T1D rats. In

Table 1
Metabolic, morphometric, and hemodynamic measures.

	ND	T1D	T1D + Th	T1D + Dize	T1D + Com
Plasma Biochemical Parameters					
PGL (mmol/L)	6.6 ± 1.1	21.7 ± 5.1 *	17.1 ± 3.8	18.8 ± 4.6	15.9 ± 3.6 $ @
CK-MB (IU/L)	23.5 ± 5.3	88.2 ± 18.4 *	56.9 ± 15.1	50.4 ± 15.3	32.6 ± 12.8 $ @
LDH (IU/L)	21.4 ± 12.4	69.4 ± 16.4 *	53.1 ± 16.0	42.6 ± 11.7	36.5 ± 13.1 $ @
Morphometric measures					
BW (g)	221 ± 52.4	163 ± 36.1 *	172 ± 42.3	174 ± 20.8	184 ± 28.4
HW (mg)	692 ± 70.5	570 ± 84.9 *	600 ± 115.2	594 ± 66.8	605 ± 60.6
HW(mg)/BW(g)	3.16 ± 0.28	3.51 ± 0.48 *	3.46 ± 0.65	3.39 ± 0.22	3.27 ± 0.28 $
Systemic blood pressure parameters					
SBP (mmHg)	117.2 ± 8.5	92.4 ± 5.6 *	97.8 ± 6.3	101.1 ± 10.3	111.3 ± 10.8 $ @
DBP (mmHg)	84.1 ± 3.8	58.1 ± 8.1 *	57.6 ± 7.9	64.2 ± 14.4	76.2 ± 9.9 $
MAP (mmHg)	95.0 ± 7.3	71.3 ± 8.4 *	75.0 ± 11.2	79.6 ± 11.6	88.9 ± 14.1 $
HR (BPM)	337 ± 31.9	235 ± 29.3 *	291 ± 33.3 $	292 ± 38.9 $	298 ± 15.7 $
Left-ventricular functional measures					
LVSP (mmHg)	111.8 ± 9.7	80.4 ± 13.7 *	86.8 ± 8.4	98.2 ± 6.6 $	103.1 ± 8.5 $ @
LVEDP (mmHg)	1.70 ± 1.3	8.02 ± 1.2 *	3.64 ± 2.3 $	3.86 ± 1.1 $	2.22 ± 0.9 $
max-dp/dt (mmHg/s)	4651 ± 682	2423 ± 504 *	3797 ± 634 $	3693 ± 761 $	4201 ± 882 $
min-dp/dt (mmHg/s)	−3721 ± 564	−2234 ± 250 *	−2721 ± 510 $	−2879 ± 789 $	−3359 ± 680 $

PGL- plasma glucose; **CK-MB-** creatine kinase-MB; **LDH-** lactate dehydrogenase; **BW**- body weight; **HW-** heart weight; **SBP-** systolic blood pressure; **DBP-** diastolic blood pressure; **MAP-** mean arterial pressure; **HR-** heart rate; **LVSP**- left-vertical systolic pressure; **LVEDP-** left-vertical end-diastolic pressure; **max-dp/dt-** rate of left-vertical pressure rise; **min-dp/dt-** rate of left-vertical pressure decline. **Note:** All the values are represented as mean ± SD; n = 8. (*) $p < 0.05$ *vs.* ND; ($) $p < 0.05$ *vs.* T1D; (@) $p < 0.05$ *vs.* T1D + Th.

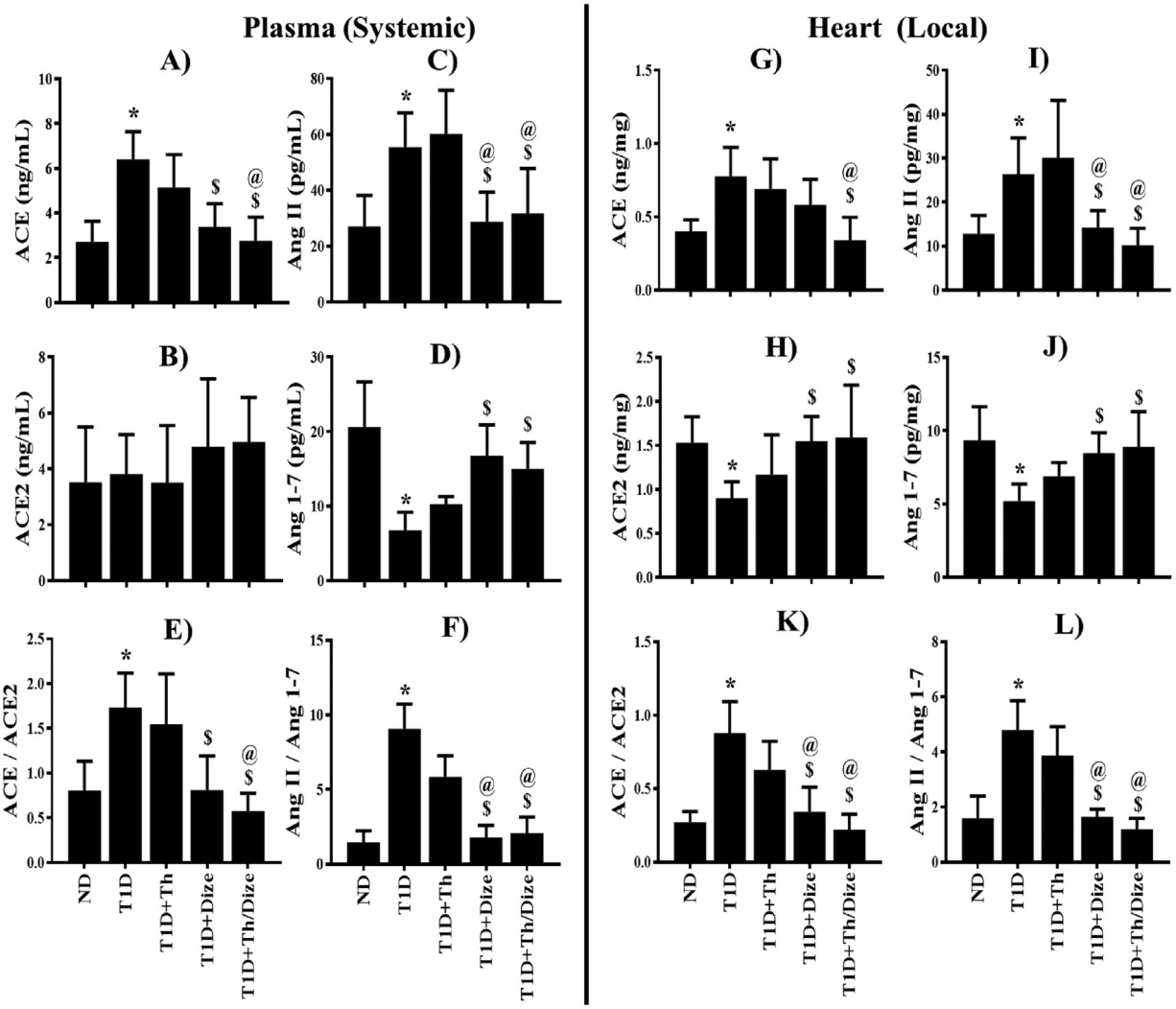

Fig. 1. Systemic and local protein levels of renin-angiotensin system components. Bar graphs A–D and G–J sequentially represented protein levels of ACE, ACE2, Ang-II and Ang-(1-7)(1–7) in plasma and heart, measured using commercially available ELISA kits. Likewise, bar graphs E–F and K–L displayed protein ratio of ACE/ACE2 and Ang-II/Ang-(1–7) in plasma and heart, respectively. All the values are represented as mean ± SD (n = 6). One-way ANOVA with Tukey's multiple comparisons test, where (*) $p < 0.05$ *vs.* ND; ($) $p < 0.05$ *vs.* T1D; (@) $p < 0.05$ *vs.* T1D + Th; (&) $p < 0.05$ *vs.* T1D + Dize.

heart, Dize monotherapy and combination therapy reduced Ang-II levels when compared to T1D and thiorphan monotherapy, and increased ACE2 and Ang-(1–7) levels when compared to T1D rats. Interestingly, combination therapy showed a significant reduction in heart ACE levels when compared to T1D and thiorphan monotherapy receiving rats (Fig. 1G–J). Moreover, in plasma and heart, T1D rats demonstrated increased ACE/ACE2 and Ang-II/Ang-(1–7) protein ratio when compared to ND rats, which were markedly abridged by Dize monotherapy and combination therapy when compared to T1D and thiorphan monotherapy (except plasma ACE/ACE2 by Dize monotherapy) (Fig. 1E–F and K–L).

Effect of thiorphan and Dize combination therapy on systemic and local NPS components levels in T1D rats

T1D rats exhibited increased plasma and heart protein levels of ANP and BNP when compared to ND rats. Thiorphan or Dize monotherapy exhibited no change in plasma and heart natriuretic peptides levels, whereas combination therapy reduced plasma ANP and BNP, and heart ANP with no change in heart BNP levels when compared to T1D rats. Interestingly, reduction in plasma BNP and heart ANP levels by combination therapy was significant when compared to thiorphan monotherapy (Fig. 2A–B and E–F). Moreover, in plasma and heart, T1D rats showed increased NEP levels when compared to ND rats. All three treatments demonstrated no change in plasma NEP level, while thiorphan monotherapy and combination therapy significantly reduced heart NEP levels when compared to T1D (Fig. 2C and G).

Thiorphan and Dize Combination therapy increased systemic and local cGMP levels in T1D rats

Plasma cGMP level was significantly reduced, and heart cGMP level remained unchanged in T1D rats when compared to ND rats. Both monotherapies and combination therapy increased plasma and heart cGMP level when compared to T1D rats. Interestingly, we observed a more pronounced increase in systemic and local cGMP levels in rats subjected to combination therapy when compared to respective monotherapies and this increase was above ND level (Fig. 2D and H).

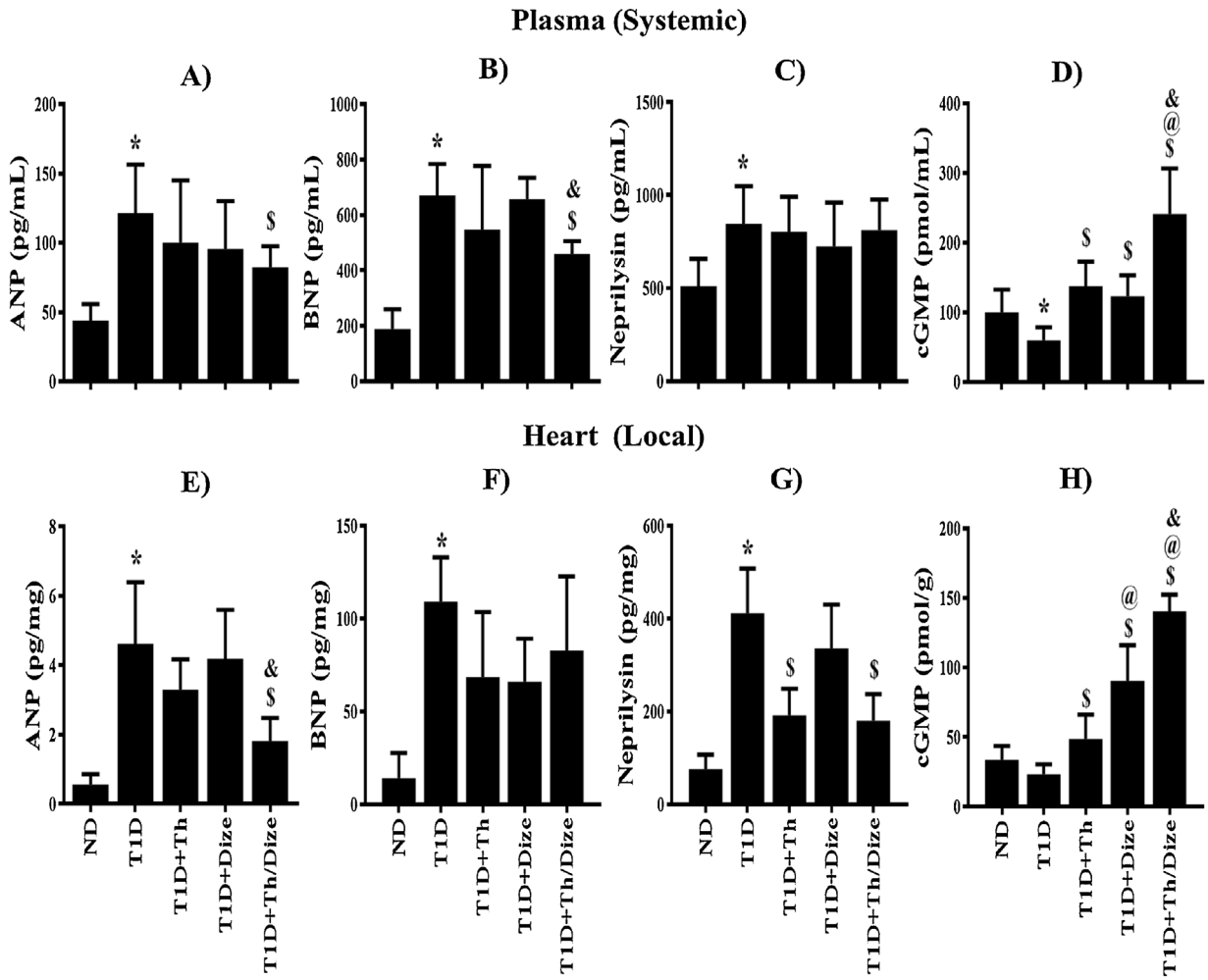

Fig. 2. Systemic and local protein levels of natriuretic peptide system components. Bar graphs A–D and E–H serially represented ANP, BNP, neprilysin, and cGMP protein levels in plasma (systemic) and heart, measured using commercially available ELISA kits. All the values are represented as mean ± SD (n = 6). One-way ANOVA with Tukey's multiple comparisons test, where (*) $p < 0.05$ *vs.* ND; ($) $p < 0.05$ *vs.* T1D; (@) $p < 0.05$ *vs.* T1D + Th; (&) $p < 0.05$ *vs.* T1D + Dize.

Thiorphan and Dize combination therapy prevented T1D associated cardiac fibrosis

In heart, T1D rats exhibited increased TGF-β (profibrotic marker) and decreased Smad7 (a negative regulator of TGF-β) expressions when compared to ND rats. Monotherapies did not change TGF-β and Smad7 expressions when compared to T1D heart. Interestingly, in heart combination therapy significantly reduced TGF-β expression when compared to T1D rats and increased Smad7 expression when compared to T1D and monotherapies receiving rats (Fig. 3A–C). Consequent with activation of profibrotic TGF-β signalling, PSR stained microscopical image analysis revealed that T1D rats exhibited increased cardiac fibrosis when compared to ND rats. Monotherapies did not affect cardiac fibrosis, whereas combination therapy significantly reduced cardiac fibrosis when compared to T1D rats (Fig. 2D–E).

Thiorphan and Dize combination therapy attenuated inflammation and apoptosis linked to DCM

In heart, T1D rats had activated inflammatory signalling evinced by increased p-NFκ-β(S-536) and p-IκBα(S-32) expressions when compared to ND rats. Thiorphan monotherapy resulted in no change in inflammatory proteins expressions, whereas Dize monotherapy reduced p-NFκ-β(S-536) expression significantly with no change in p-IκBα(S-32) expression. In contrast, rats receiving combination therapy showed a significant reduction in p-NFκ-β(S-536) and p-IκBα(S-32) expressions in the heart compared to T1D rats (Fig. 4A–C). Moreover, T1D rats showed increased cardiac cell apoptosis as revealed by increased expressions of c-PARP and c-Caspase-3 in the heart when compared to ND rats. Thiorphan and Dize monotherapy reduced c-PARP expression, while only Dize monotherapy reduced c-Caspase-3 expression when compared to T1D rat's hearts. Interestingly, combination therapy showed a more pronounced reduction in apoptotic markers expressions in the heart when compared to T1D and thiorphan monotherapy receiving rats (Fig. 4A and D–E).

Thiorphan and Dize combination therapy abridged H3K4Me2 and histone methyltransferases (HMTs) expressions in DCM

T1D rats' heart demonstrated increased RBBP5 and SET7/9 and reduced SET8 expressions when compared to ND rats. Thiorphan monotherapy significantly reduced RBBP5 expression, while not altering SET7/9 and SET8 expressions in the heart when compared to T1D rats. In contrast, Dize monotherapy significantly reduced SET7/9 and increased SET8 with no change in RBBP5 expression in the heart when compared to T1D rats. Interestingly, combination therapy markedly reduced RBBP5 and SET7/9 and increased SET8 expression in the heart when compared to T1D rats (Fig. 4A and

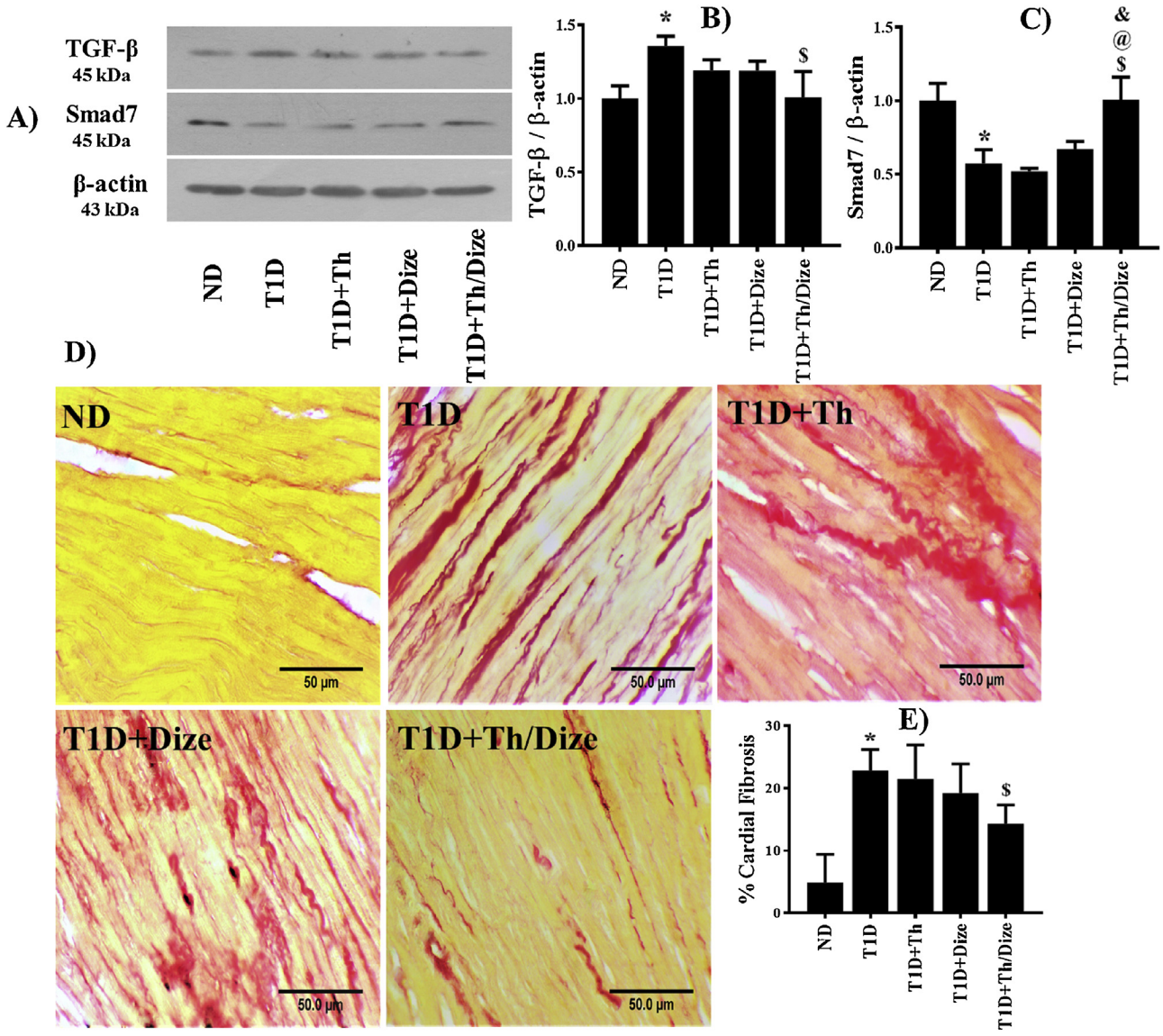

Fig. 3. Thiorphan and Dize combination therapy prevented cardiac fibrosis. A) Representative immunoblots for profibrotic markers TGF-β and Smad7 expressions in the heart. Fold change in protein expressions of B) TGF-β and C) Smad7 in rats' heart of various study groups when compared to ND rats. β-actin expression was used as a loading control. D) microscopical images of Picrosirius red (PSR) stained heart sections portraying cardiac fibrosis (original magnification 1000× and Scale bar – 50 µm). Captured images were semi-quantitively analysed by ImageJ software to calculate percentage PSR positive area (cardiac fibrosis marker), and final analysis is represented as bar graph E) showing percentage cardiac fibrosis. All the values are represented as mean ± SD (n = 3). One-way ANOVA with Tukey's multiple comparisons test, where (*) $p < 0.05$ *vs.* ND; ($) $p < 0.05$ *vs.* T1D; (@) $p < 0.05$ *vs.* T1D + Th; (&) $p < 0.05$ *vs.* T1D + Dize.

E–G). In line with HMTs expressions, T1D rats' hearts exhibited significantly increased H3K4Me2 expression when compared to ND rats. Monotherapies did not affect H3K4Me2 expression, while combination therapy significantly reduced H3K4Me2 expression in the heart when compared to T1D rats and both monotherapies (Fig. 4A and H).

Discussion

Away from traditional tactics of treating DCM by conventional RAS blockade (*e.g.* ARB and ACEi), either as monotherapy or as combination therapy with NEPi (*e.g.* vasopeptidase inhibitors and ARNi), this investigation aimed at evaluating the efficacy of NEPi and ACE2 activator combination against streptozotocin-induced DCM in male Wistar rats. T1D rats demonstrated an array of symptoms confirming the development of DCM, including biochemical and morphometric alterations, systemic cardiac and left ventricular functional decline. Thiorphan and Dize combination therapy markedly attenuated metabolic perturbations and alleviated cardiovascular functions in T1D rats (Table 1). Moreover, T1D rats exhibited unfavourable alterations in systemic (plasma) and local (heart) RAS [ACE, ACE2, Ang-II and Ang-(1–7)] NPS (ANP, BNP, and NEP) components. As we targeted NPS and RAS simultaneously using NEPi and ACE2 activator combination therapy, it normalised these changes (Figs. 1 and 2). Interestingly, combination therapy resulted in inhibition of inflammatory, profibrotic and apoptotic signalling cascades in T1D rats (Figs. 3 and 4). Recently, we have reported the role of H3K4Me2 and H3K4 methyltransferase (SET7/9) in the development of diabetic renal fibrosis [9]. In this regard, T1D rats' hearts showed increased H3K4Me2 and H3K4 methyltransferase RBBP5 and SET7/9 expressions, which were normalised by combination therapy (Fig. 4). In short, cardiovascular protection achieved by thiorphan and Dize combination therapy was superior to respective monotherapies. However, if we compare the monotherapies than Dize monotherapy found better in attenuating the development of

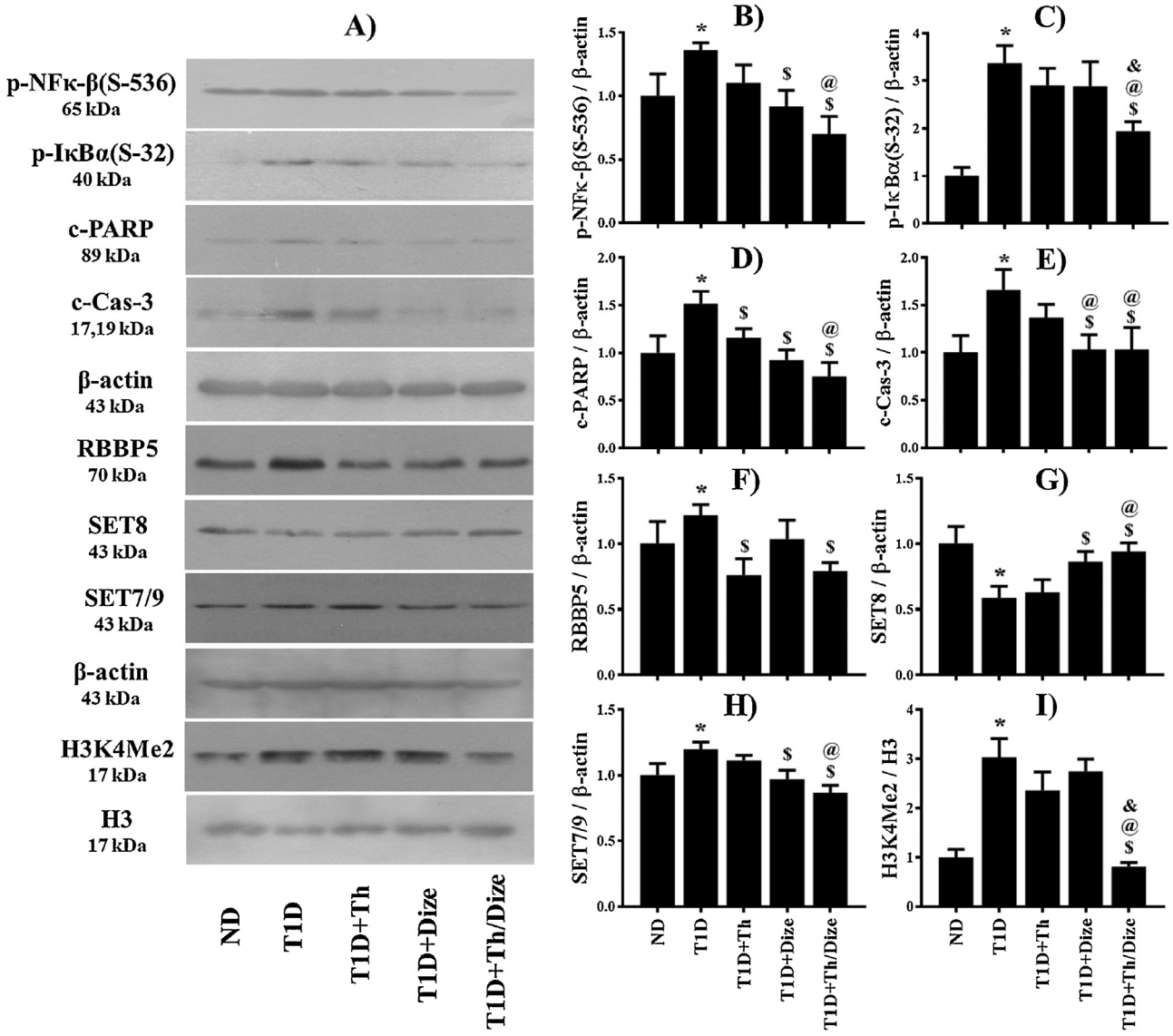

Fig. 4. Thiorphan and Dize combination therapy attenuated inflammation and apoptosis, and normalised histone methylation. A) represents, immunoblots for inflammatory [p-NFκ-β(S-536) and p-IKβα(S-32)] and apoptotic (c-PARP and c-Caspase-3) markers, histone H3 methyltransferases (RBBP5, SET7/9 and SET8) and corresponding histone posttranslational modification H3K4Me2 expressions in the heart. Respective β-actin or H3 blots were used as a loading control. Bar graphs, B–H sequentially represents fold change in protein expressions p-NFκ-β(S-536), p-IKβα(S-32), c-PARP, c-Caspase-3, RBBP5, SET7/9, SET8 and H3K4Me2 in rats' heart of various study groups when compared to ND rats. All the values are represented as mean ± SD (n = 3). One-way ANOVA with Tukey's multiple comparisons test, where (*) $p < 0.05$ *vs.* ND; ($) $p < 0.05$ *vs.* T1D; (@) $p < 0.05$ *vs.* T1D + Th; (&) $p < 0.05$ *vs.* T1D + Dize.

DCM when compared to thiorphan monotherapy, which was close to combination therapy.

Hyperglycaemia is the root cause for diabetic microvascular and macrovascular complications, including DCM [1,18]. In line with our previous report, here we observed a substantial reduction in hyperglycaemia by thiorphan and Dize combination therapy, whereas monotherapies did not affect hyperglycaemia in T1D rats [15]. Combination therapy reduced plasma levels of CK-MB and LDH (circulating molecular markers for cardiac damage) in T1D rats. In contrast, all three therapeutic regimens failed to alleviate morphometric alterations associated with diabetes, with the exception to HW/BW ratio (cardiac hypertrophy index), which was significantly reduced by combination therapy in T1D rats. Combination therapy also improved systemic blood pressure and left ventricular function parameters, which are considered as gold standards to assess cardiac functionality (Table 1) [8].

In diabetic individuals, the pathological fate of DCM is determined by the status of systemic and local RAS and NPS [1]. In the present study, T1D rats showed unfavourable changes in systemic and local RAS-like reduced beneficial Ang-(1–7) and ACE2 (only local) levels and increased detrimental ACE and Ang-II level, ACE/ACE2 and Ang-II/Ang-(1–7) ratio. Similarly, T1D rats had increased systemic and local NEP, ANP and BNP levels. The therapeutic interventions used in the present study, thiorphan and Dize reported to increases the bioavailability of natriuretic peptides and stimulated the depressor arm of RAS; respectively, both eventually will benefit cardiovascular system [8,9,12,19]. Recently, we reported that thiorphan and Dize combination therapy normalised intrarenal RAS in T1D rats [15]. In the present study, thiorphan and Dize combination therapy significantly alleviated systemic and local RAS and NPS perturbations in T1D rats (Figs. 1 and 2).

The cGMP is a secondary messenger generated *via* activation of receptor enzymes called particulate guanylyl cyclase (pGC); NPR-A is one of them, and natriuretic peptides act as ligands [14]. Also, endothelial NO synthase-derived NO stimulates soluble GC (sGC) and eventually produce cGMP from GTP [20]. The cGMP has been reported to have several beneficial effects on cardiovascular. Hence deficit in cGMP level could manifest in CVD [14,21–23]. Here, T1D rats exhibited a significant reduction in systemic cGMP levels when compared to ND rats. Interestingly, therapeutic regimen target towards increasing cGMP level (*e.g.* phosphodiesterase-5 inhibitors, NEPi, recombinant natriuretic peptides, and sGC stimulator) helps in preventing CVD [14,21,23]. Considering these facts, the hypothesis behind simultaneous administration of NEPi and ACE2 activator was that, NEPi (thiorphan) will act on pGC *via* increasing natriuretic peptides bioavailability [1,24], whereas ACE2 activator (Dize) will act on sGC *via* activation of PI3K/Akt pathway [9,10,25], thus as a collective efforts will increase the cGMP levels. In support of this hypothesis, previously we observed increased cGMP levels by combination therapy in the diabetic kidney [15]. Here, we observed that thiorphan and Dize, when administered alone as monotherapy or together as combination therapy markedly increases plasma and heart cGMP levels; however, the degree of increase was significantly higher in combination therapy.

Hyperglycaemia derived activation of profibrotic, inflammatory, and apoptotic signalling are key to the development of DCM [26,27]. Hence, aiming inhibition of these pathological cascades serves as an essential therapeutic approach against DCM. T1D rats had activated profibrotic signalling indicated by increased TGF-β expression and reduced its inhibitor Smad7 expression in heart, resulted in increased cardiac fibrosis examined by PSR staining. Recent reports suggest that Dize monotherapy (15 mg/kg/day) and Dize (5 mg/kg/day) in combination with thiorphan reduced TGF-β expression in T1D rats' kidneys, thereby abridged fibrosis in preventing DN [9,15]. Similarly, in the present study T1D rats subjected to thiorphan and Dize combination therapy showed a significant reduction in TGF-β and augmentation in Smad7 expression in heart, might be a reason for the subsequent decrease in cardiac fibrosis as revealed by PSR staining (Fig. 3).

In the inactive state, NF-κB forms a complex with its inhibitory kinase IκBα and remains in the cytosol, while IκBα phosphorylation resulted into ubiquitin-mediated proteasome-dependent degradation of IκBα and the release p-NF-κB which translocate to the nucleus. Resulted activated NF-κB signalling, intensifies inflammatory response by augmenting production of proinflammatory cytokines, adhesion molecules and angiogenic mediators [27]. In the present study, T1D rats showed increased expression of p-NFκ-β(S-536) and p-IκBα(S-32) in the heart when compared to

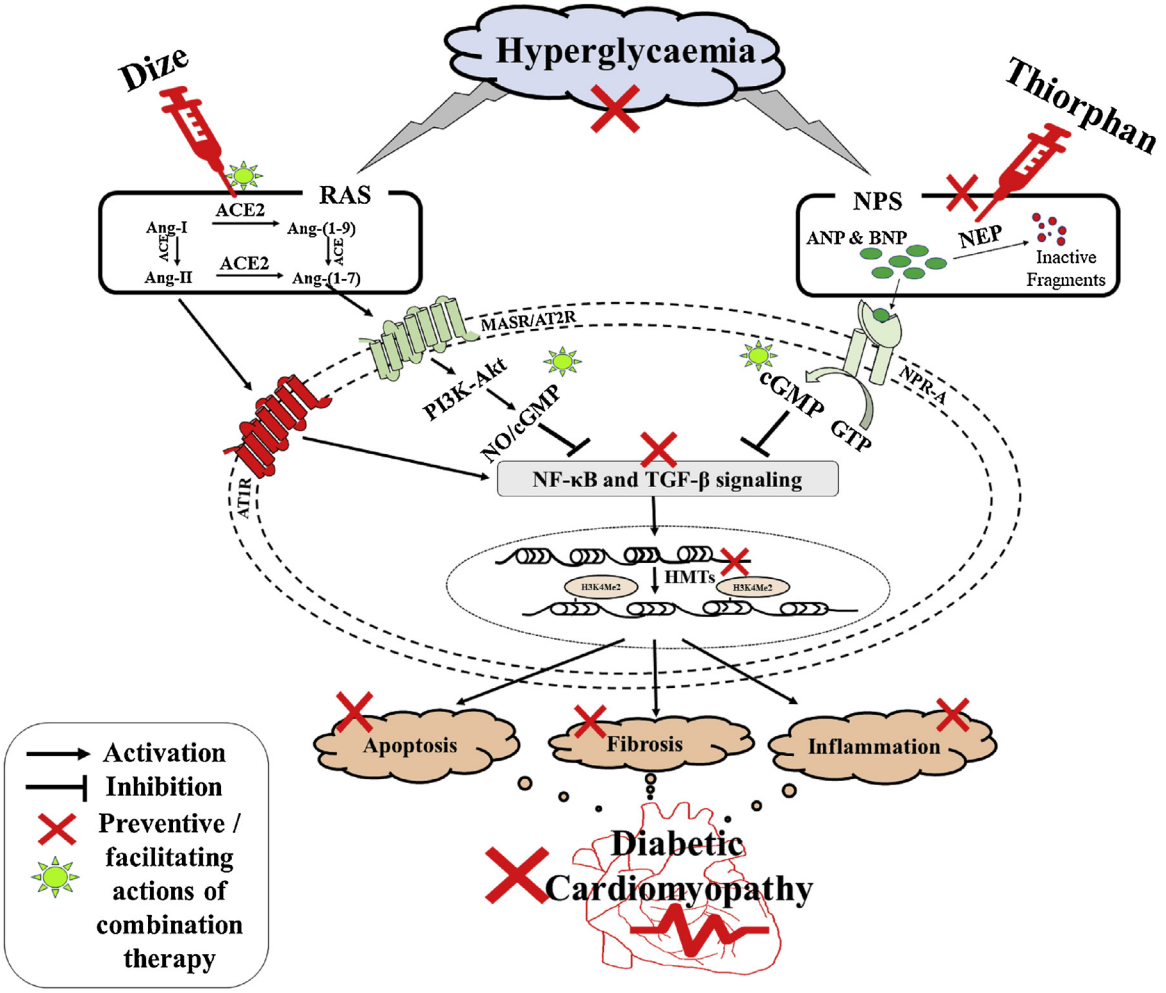

Fig. 5. Diagram representing the mechanism of action of thiorphan and Dize combination in preventing DCM. Proposed combination therapy of thiorphan (neprilysin inhibitor) and Dize (ACE2 activator) prevented hyperglycaemia and other metabolic alterations, normalized natriuretic peptide and renin-angiotensin system components levels, increased cGMP level, inhibited inflammation, fibrosis and apoptosis, abridged histone methyltransferases (HMTs) levels and revered H3K4Me2 levels, eventually prevented diabetic cardiomyopathy in streptozotocin-induced diabetic male Wistar rats.

ND rats. Recently, we have demonstrated that thiorphan in combination with Dize inhibited activation NF-κB signalling in preventing DN [15]. Consistent with this, here we observed a significant reduction in p-NFκ-β(S-536) and p-IκBα(S-32) expression in rats' hearts receiving combination therapy. Also, T1D rats exhibited increased expression of apoptotic markers (*e.g.* c-PARP and c-Caspase-3) in the heart which was substantially reduced by thiorphan and Dize combination therapy. Taken together, the present study results revealed that combination therapy of thiorphan and Dize halted activation of inflammatory, profibrotic and apoptotic signalling way better than respective monotherapies in attenuating DCM.

The accumulated body of evidence suggests that genetic penchant alone will explain the pathogenesis of DCM incompletely. Thus we should look into epigenetic mechanisms involved in the same [28]. Histone posttranslational modifications (PTM) are one of the most widely studied epigenetic alterations. Moreover, existing literature established a strong correlation between TGF-β and NF-κB signalling activation and modulation of histone methylation and methyltransferases expressions in diabetes [17,28–30]. *In vitro* studies demonstrated that histone methyltransferase SET7/9 mediates high glucose-induced inflammation *via* epigenetic regulation of the transcription factor NF-kB, similar results were obtained from T1D patients [31,32]. Recently, we demonstrated the functional role of H2A and H2B mono-ubiquitination in regulating H3K4Me2 through modulation of SET7/9 expression in the development of diabetic renal fibrosis [17]. Hence, in the present study, we explored H3K4Me2 and its methyltransferases expressions in DCM. In the heart, T1D rats showed increased H3K4Me2, RBBP5 and SET7/9, and reduced SET8 expressions, while combination therapy significantly attenuated these epigenetic alterations. Hence, a reversal of H3K4Me2 status by combination therapy, at least in part, contribute to its beneficial effect against DCM.

Conclusion

In streptozotocin-induced DCM, superior cardio-protection achieved by NEPi (thiorphan) and ACE2 activator (Dize) combination therapy over respective monotherapies is largely attributed to attenuation of metabolic and morphometric (partially) alterations, normalization of systemic and local RAS and NPS components, improvement in cGMP levels, inhibition of profibrotic, inflammatory, and apoptotic signalling cascades, and reversal of H3K4Me2 status (Fig. 5). In summary, this is, to the best of our knowledge, the first study comprehensively revealing the protective effects of NEPi and ACE2 activator combination against DCM.

Author contributions

V.M. and A.B.G. conceived the idea and designed the experiments, V.M. and N.S. performed all the experiments and analyzed the data, V. M. completed the manuscript writing and A.B.G. reviewed and revised the manuscript for important intellectual content.

Conflict of interest

The authors declare that there is no conflict of interest associated with this manuscript.

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