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**Inhibition of endoplasmic reticulum stress combined with activation of angiotensin-
converting enzyme 2: Novel approach for the prevention of endothelial dysfunction in type
1 diabetic rats**

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Abstract: Persistent hyperglycemia in type 1 diabetes triggers numerous signaling pathways, which may prove deleterious to the endothelium. Since hyperglycemia damages the endothelial layer via multiple signaling pathways, including enhanced oxidative stress, downregulation of angiotensin-converting enzyme2 signaling, and exacerbation of endoplasmic reticulum stress, etc.; hence it becomes difficult to prevent the injury using monotherapy. Thus, the present study was conceived to evaluate the combined effect of endoplasmic reticulum stress inhibition along with angiotensin-converting enzyme-2 activation, two major contributors to hyperglycemia-induced endothelial dysfunction, in preventing endothelial dysfunction associated with type 1 diabetes. Streptozotocin-induced diabetic animals were treated with either diminazene aceturate (5 mg kg-day⁻¹, *p.o.*) or tauroursodeoxycholic acid, sodium salt (200 mg kg- day⁻¹ *i.p.*), or both for four weeks. Endothelial dysfunction was evaluated using vasoreactivity assay, where acetylcholine-induced relaxation was assessed in phenylephrine pre-contracted rings. Combination therapy significantly improved vascular relaxation when compared to diabetic control as well as monotherapy. Restoration of nitrite levels along with prevention of collagen led to improved vasodilatation. Moreover, there was an overall reduction in aortic oxidative stress. We conclude that by simultaneously inhibiting ER stress and activating angiotensin-converting enzyme-2 deleterious effects of hyperglycemia on endothelium were significantly alleviated. This could serve as a novel strategy for the prevention of endothelial dysfunction.

Keywords: Endothelial dysfunction; Endoplasmic reticulum stress; Angiotensin-converting enzyme-2; Type-1 diabetes.

45 **Introduction**

46 Diabetes mellitus leads to different macrovascular complications, which are the major contributors
47 to the morbidity and mortality associated with diabetes (Bjornstad et al. 2018; Varma et al. 2005).
48 This could be primarily attributed to the endothelial dysfunction, which results due to the elevated
49 oxidative stress and endoplasmic reticulum (ER) stress owing to the persistent hyperglycemia
50 (Maamoun et al. 2019; Rajendran et al. 2013). Moreover, endothelial dysfunction is considered an
51 important target to prevent the progression of cardiovascular disorders (Versari et al. 2009). The
52 hallmark and primary reason behind endothelial dysfunction development is the reduced
53 bioavailability of nitric oxide (NO). Several pathophysiological conditions may reduce the NO
54 levels, hyperglycemia being one of them (Chen et al. 2018). Also, persistent hyperglycemia is well
55 known to alter the pressor arm of the renin-angiotensin system and increase the levels of
56 angiotensin-II, which in turn increases the oxidative stress (Lavrentyev et al. 2007; Pueyo et al.
57 2000) and ER stress in endothelial cells (Liang et al. 2013; Murugan et al. 2015). ER stress could
58 suppress the eNOS expression, thus reducing the NO level in the aortic rings obtained from mice
59 (Lau et al. 2018). Studies even suggest a probable mechanistic link between ER stress and
60 oxidative stress being implicated in cardiovascular disorders associated with diabetes, probably
61 due to both being important mediators of endothelial dysfunction (Ochoa et al. 2018). Furthermore,
62 hyperglycemia is also known to reduce the angiotensin-converting enzyme- 2 (ACE2) levels which
63 forms the depressor arm of the renin-angiotensin system and is vasoprotective in nature. ACE2
64 knockout animals had vascular dysfunction due to reduced NO bioavailability and enhanced
65 oxidative stress (Rabelo et al. 2016). ACE2 and angiotensin (1-7) are emerging as an important
66 therapeutic target to counter the endothelial dysfunction. Direct administration of angiotensin (1-
67 7) as well as ACE2 activation both have been proven to be beneficial in abrogating endothelial

dysfunction by reducing oxidative stress and ER stress (Zhang et al. 2015). Diabetic patients consistently manifest an impairment in vasodilation due to endothelial damage. Hence, it becomes imperative to target the different molecular mechanisms behind diabetic endothelial dysfunction to avert vascular complications associated with all forms of diabetes mellitus. Since diabetic endothelial dysfunction is of multiple etiology so targeting, different signaling pathways simultaneously could be a better approach. Therefore, we hypothesized that simultaneous inhibition of endoplasmic stress and activation of ACE2 by novel combination of diminazene aceturate (ACE2 activator) and tauroursodeoxycholic acid (ER stress inhibitor) respectively might produce better vasoprotection and could prevent endothelial dysfunction.

Materials and Methods:

Drugs

Streptozotocin and Diminazene aceturate were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Tauroursodeoxycholic acid (TUDCA), sodium salt, was purchased from Merck [Darmstadt, Germany].

Animal studies and drug treatment

Male adult Wistar rats (200–220 g) were procured from the central animal facility of Birla Institute of Technology and Science Pilani (BITS Pilani) and were allowed to acclimatize in standard environmental conditions for a week. Animals were provided with a standard pellet diet and water ad libitum.

The study was performed after the protocol approval by the Institutional Animal Ethics Committee (IAEC), BITS, Pilani [IAEC/RES/25/14/Rev-1/27/15]. After the acclimatization period, type 1 diabetes was induced in the animals as described by Goru et al. (Goru et al. 2017). Briefly, type 1

diabetes was induced by injecting a single dose of STZ [55 mg/kg, *i.p.*, dissolved in ice-cold sodium citrate buffer (0.01 M, pH 4.4)]. Normal control rats were administered only sodium citrate buffer. Post 48 hrs. of induction, animals with plasma glucose levels (PGLs) >250 mg/dl were considered diabetic animals.

All the animals were divided into five groups of six animals each after 4 weeks of diabetes induction. (i) Normal control (NC) group received vehicle treatment. (ii) Diabetic control (DC) group (iii) DC animals were administered with diminazene aceturate ($5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, *p.o.*) (Goru et al. 2017) (iv) DC animals were administered with TUDCA ($200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, *i. p.*) (Choi et al. 2016) (v) DC animals were treated with a combination of diminazene aceturate ($5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, *p.o.*) and TUDCA ($200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, *i. p.*). The treatment was continued from week 4 to week 8. -Post-treatment, animals were sacrificed, followed by the immediate collection of thoracic aortas.

Plasma Glucose

At the end of the study, blood was collected from the animals, followed by plasma separation. Commercially available kits were used to evaluate the plasma glucose levels. The assay was based on the GOD-POD method.

Aorta oxidative stress parameters

Malondialdehyde (MDA) levels

Lipid peroxidation results due to enhanced oxidative stress. To evaluate the same, MDA production was assessed with a thiobarbituric acid (TBA) reaction in aortic homogenates from the rats. MDA is known to form a condensation product on reaction with thiobarbituric acid. Briefly, the aortic homogenate was added to freshly made thiobarbituric acid followed by incubation at

95°C for 1 hr. The mixture was further centrifuged, and the supernatant was measured spectrophotometrically at 532nm for MDA-TBA adduct. The final concentration of MDA was derived from a standard curve and expressed as nM of MDA/mg protein (Kadacol et al. 2017b).

Glutathione (GSH) assay

Reduced glutathione levels in the aortic tissues were measured as a marker for antioxidant activity. The assay was performed using 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), also known as Ellman's reagent. Initially, a standard calibration curve was obtained using reduced glutathione. Ellman's reagent was added to the aortic homogenates, followed by an incubation of 15 minutes. Plates were read at 410 nm, and the concentration of GSH was expressed as µg of GSH/mg protein (Kadacol et al. 2017b).

Vascular reactivity assay

Thoracic aorta isolation and vascular reactivity assay were performed as per the previous protocols. At the end of the study, rats were sacrificed, and the thoracic aorta was removed carefully. The aorta was further cleaned of the perivascular fat and adhering adventitial tissues. The whole procedure was carried out in ice-cold modified Krebs Henseleit buffer at pH 7.4. The aorta was then excised into rings of 5-6 mm. The aortic rings were suspended between a pair of stainless-steel stirrups and placed in a water-jacketed organ bath [Ugo Basile, Varese, Italy], which was filled with 10 ml of modified Krebs Henseleit buffer which was continuously bubbled with carbogen (95% O₂ + 5% CO₂) and the temperature being kept at 37°C. The tissue was allowed to stabilize for a period of 60 minutes. Buffer in the organ chamber was changed every 15 minutes. Post stabilization, the tissue was exposed to 80 mM of KCl to evaluate the viability and for priming the tissue. The tissue was washed and allowed to stabilize. After that, the tissue was exposed to a submaximal concentration of phenylephrine (0.3 µM) and was allowed to

contract until the peak reached a plateau. This was followed by the cumulative addition of acetylcholine (10^{-9} – 10^{-4} M). The response was taken on the Powerlab data acquisition system using the software LabChart 7 Pro (AD Instruments, Australia) (Kadacol et al. 2017a).

Nitrite level estimation

Nitrite levels are vital to the preservation of vascular tone. We measured the total nitrite in the aortic homogenate using the Griess assay. Nitrate was reduced to nitrite in the samples since nitrates are highly unstable and difficult to measure. This was followed by mixing of the sample with Griess reagent (0.1% N-(1- naphthyl) ethylenediamine dihydrochloride, 1 % sulfanilamide, and 2.5 % phosphoric acid). The mixture was further incubated in the dark for 30 minutes. Spectrophotometric absorbance was recorded at 540 nm. Tissue nitrite levels were calculated using the standard curve developed using geometric dilutions of sodium nitrite and expressed as $\mu\text{mol/mg}$ of protein (Sharma and Gaikwad 2020).

Picrosirius red staining

Picrosirius red staining is a histological technique that is employed to assess collagen deposition in a paraffin-embedded tissue block. It was performed as per the previously established laboratory protocols. Briefly, the aortic tissue was fixed in 10 % (v/v) for 72 hrs. Followed by paraffin embedding. Tissue sections of 6 μm thickness were obtained. Later the sections were stained with picrosirius red dye in order to quantify the collagen. A minimum of 4-5 sections per slide and 6 tissue slides from each group were observed. Images were captured using a Zeiss Primostar microscope at 400x magnification. The images were further analyzed using Image J software for the measurement of %PSR positive area (collagen deposition) (Kadacol et al. 2017a).

Statistical analysis

All the experimental values are expressed as mean $\pm$ SEM ($n = 6$). Graph pad PRISM 5 software (Graph pad Software Inc., San Diego, California, USA) was used for statistical calculations. One-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test was used for statistical comparison, and the 'F' value was considered significant if $p < 0.05$. The pD₂ value (-log EC₅₀) was calculated from the concentration-response curve by non-linear regression analysis of the curve.

Ethics approval

Animal studies were performed as per the guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, Government of India. Further, all the animal protocols were approved by the Institutional Animal Ethics Committee (IAEC), BITS, Pilani (Approval No.: IAEC/RES/25/14/Rev-1/27/15). For the preparation of this manuscript, the ARRIVE 2.0 (Animal Research: Reporting of In Vivo Experiments) guidelines were followed.

Results

Diminazene aceturate and TUDCA combination therapy normalized the blood glucose levels

Type 1 diabetic rats were significantly hyperglycemic as compared to NC rats. Treatment with TUDCA effectively reduced the plasma glucose levels however, diminazene aceturate monotherapy does not reduce the blood glucose levels. Moreover, the combination therapy was found to significantly reduce the blood glucose levels when compared to the DC group as well as monotherapies [Fig. 1a].

Diminazene aceturate and TUDCA combination therapy reduced the aortic oxidative stress

The levels of glutathione were drastically reduced in the aortic tissue of streptozotocin-treated animals, along with increased MDA levels indicating higher lipid peroxidation. This is an indication of increased oxidative stress due to hyperglycemia. Further, treatment with diminazene aceturate, TUDCA, and their combination for 4 weeks successfully restored the glutathione levels and reduced the lipid peroxidation as depicted in Fig. (1b; 1c). However, the combination therapy brought a significant reduction in the MDA levels as compared to monotherapies.

Combination therapy of diminazene aceturate and TUDCA improved the aortic vasorelaxation

Impairment in vasorelaxation is the primary cause behind the development of endothelial dysfunction. During vasoreactivity assay, the phenylephrine pre-contracted aorta when exposed to Ach cumulatively produced dose-dependent vasorelaxation in all the groups. However, the relaxation produced in the aorta from the DC group was minimal and significantly lower than the rest of the groups. Monotherapies *per se* were able to improve the vasorelaxation significantly when compared to diseased animals' aorta. But the vasorelaxation produced by the combination therapy was significantly better than that of monotherapies and almost equivalent to the vasorelaxation produced in the normal animal aorta [Fig. 2]. The pD_2 values and E_{max} are provided in table 1.

Combination therapy of diminazene aceturate and TUDCA restored the aortic nitrite levels

Total nitrite was significantly lower in the diabetic group aorta in comparison to the normal control animals' aorta. Nitrite level restoration was observed post the treatment of TUDCA and diminazene aceturate for 4 weeks. Interestingly, the levels observed in the groups being

administered the combination therapy were significantly better when juxtaposed to monotherapies [Fig. 3].

Simultaneous activation of ACE2 and inhibition of ER stress reduced the aortic collagen deposition

Collagen deposition in the aorta leads to stiffness and consequent loss in the vasorelaxation of the blood vessel. PSR stained images of the thoracic aorta sections revealed a massive collagen deposition in the aorta of DC rats, as can be observed by the color intensity. Monotherapies with Dize and TUDCA reduced the collagen levels significantly when compared with diseased control. Moreover, the effect produced by the combination of these drugs was even better, and the collagen level was found to be normalized and significantly lower than the DC group as well as monotherapies [Fig. 4].

Discussion and Conclusion

Vascular endothelial dysfunction is defined as the reduced vasorelaxation and enhanced vasoconstriction of the blood vessels (Sankrityayan and Majumdar 2016). Several factors could influence vascular homeostasis leading to the development of endothelial dysfunction. The endothelial layer is paramount to the homeostasis in the vascular biological system since they line the entire circulatory system and act as a physical barrier along with the provider of crucial regulatory substances (Pi et al. 2018). The majority of the cardiovascular complications due to diabetes could be attributed to the damage to the endothelial layer by the mediators produced owing to persistent hyperglycemia (Sena et al. 2013). Hyperglycemia leads to the alteration of the renin-angiotensin system characterized by elevated angiotensin-II, which is a potent vasoconstrictor and a well-known inducer of oxidative and ER stress. Besides, during

221 hyperglycemia, the protective arm of the renin-angiotensin system consisting of ACE2 and
222 angiotensin (1-7) is suppressed (Senanayake et al. 2018). Previously, angiotensin (1-7) has been
223 found to induce vasodilatation via different mechanisms (Murugan et al. 2015; Zhang et al. 2015).
224 Damage to the vascular endothelium during diabetes is of multiple etiology and hence it is difficult
225 to prevent it using a single therapy.

226 Here in this study, we have used a combination therapy consisting of an ACE2 activator and ER
227 stress inhibitor to counter the hyperglycemic damage to endothelium in a better way.
228 Streptozotocin-induced type 1 diabetic rats showed a significant increase the blood glucose. In
229 monotherapies, TUDCA was found to reduce blood glucose levels. This was in concordance with
230 previous studies where ER stress inhibition using TUDCA has resulted in reduced blood glucose
231 levels (Bronczek et al. 2019; Ozcan et al. 2006). Bronczek et al. attributed the glucose-lowering
232 effect of TUDCA to inhibition of hepatic insulin-degrading enzyme and increasing the activity of
233 this enzyme, thus causing an improvement in the activity of the insulin. Moreover, they also
234 reported that TUDCA has a β cell-protective property, and it increases the β cell number as well
235 as its mass, explaining the anti-hyperglycemic property of TUDCA (Bronczek et al. 2019). In fact,
236 a recent study showed that persistent ER stress could promote hyperglycemia (Liu et al. 2019).
237 Although, we did not find a significant change in the blood glucose level post ACE2 activation.
238 However, when it was combined with TUDCA, the combination showed additional glucose-
239 lowering ability. Few studies have even reported that ACE2 reduced hyperglycemia owing to
240 properties such as reduced oxidative stress, increased blood flow to the β cells of the pancreas
241 (Chhabra et al. 2013). This could explain the additional anti-hyperglycemic effect of the
242 combination. Impairment in the vasorelaxation is the most important characteristic of endothelial
243 dysfunction.

244 We also found that prolonged hyperglycemia led to the reduction of Ach-induced vasorelaxation
245 in the aorta. This was in line with previous studies where hyperglycemia has led to reduced
246 vasorelaxation (Romero et al. 2008; Sun et al. 2017). Multiple reasons have been proposed for the
247 same, including elevated oxidative stress and ER stress which leads to the damage of endothelium
248 and reduction in the bioavailability of NO (Imanishi et al. 2006). ER stress inhibition could be
249 beneficial in endothelial dysfunction because they have the capacity to restore the bioavailability
250 of NO, an important factor in maintaining vascular homeostasis. In our study, we found that
251 treatment with TUDCA improved the levels of NO in the aorta of diabetic rats. Studies also suggest
252 that ER stress may induce endothelial cell apoptosis and increase oxidative stress, both responsible
253 for endothelial dysfunction. Also, hyperglycemia may lead to elevated oxidative stress due to
254 increased angiotensin-II and reduced ACE2 and angiotensin (1-7) activity. Such an increase in
255 oxidative stress due to elevated angiotensin-II is also known to reduce NO bioavailability. This
256 was supported by our results where ACE2 activation using diminazene aceturate markedly reduced
257 the oxidative stress levels as observed by improved glutathione and reduced MDA levels. Due to
258 the interlink pathophysiology of ER stress and oxidative stress, we thought of inhibiting both using
259 a combination of drugs. Consequently, we observed that the combination therapy was better than
260 monotherapies in alleviating oxidative stress and elevating the NO bioavailability. Aortic
261 stiffening is also a major contributor to the loss of the vasorelaxant ability of the aorta (Grigorova
262 et al. 2015). Diabetes leads to the deposition of excess collagen in the aorta. In this study, PSR
263 staining showed excessive collagen in the diabetic rat aorta in comparison to that of the normal rat
264 aorta. Moreover, we observed a significant reduction in the collagen deposition in the animals
265 treated with ACE2 activator and ER stress inhibitor. Hyperglycemia-induced elevation in
266 angiotensin-II and consequential ER stress might be a reason behind the collagen deposition in the

aorta. Earlier reports corroborate the same where sustained ER stress due to angiotensin-II infusion led to collagen deposition (Spitler and Webb 2014). So, activating ACE2 will counteract the effect produced on the blood vessel by angiotensin-II signaling. Simultaneously, administration of TUDCA will directly reduce the ER stress resulting in inhibition of the pro-apoptotic and pro-fibrotic signaling pathways, thus arresting the collagen deposition. Hence, we conclude that the simultaneous inhibition of ER stress and ACE2 restored the endothelial function more significantly by reducing oxidative stress, improving NO bioavailability, and reducing collagen deposition. Although, the pharmacological agents used in the study have been found to modulate the ER stress and ACE2 levels previously in several similar studies. The current study suffers from a potential limitation which is the lack of measurement of ER stress inhibition as well as ACE2 activation parameters *per se*.

This study provides a novel combination of ER stress inhibitor and ACE2 activator, which could work better in alleviating diabetic endothelial dysfunction better than the respective monotherapies. The novel combination proposed in this study could be a valuable therapeutic strategy against endothelial dysfunction.

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Conflict of interest

The authors declare no conflicts of interest.

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Figure Legends

Figure 1: Effect of different treatments on plasma glucose levels and aortic oxidative stress parameters.

All the values are represented as mean $\pm$ SEM; n=6 (p<0.05; ** vs NC; \$ vs DC; @ vs DC+Dize; # vs DC+TDC. (a) Plasma glucose levels (b) Reduced GSH levels (c) MDA levels post treatment. NC: Normal Control; DC: Diabetic control; DC+Dize: Diabetic control treated with diminazene aceturate; DC+TDC: Diabetic control treated with tauroursodeoxycholic acid; DC+ Comb.: Diabetic control treated with a combination of diminazene aceturate and tauroursodeoxycholic acid.

Figure 2: Concentration dependent relaxation of acetylcholine on aortic rings (with intact endothelium) pre-contracted with PE obtained from different treatment groups.

Tension is expressed as % relaxation on initial contraction with PE. Values are expressed as mean $\pm$ SEM. (p<0.05; * vs NC; \$ vs DC; @ vs DC+Dize; # vs DC+TDC.

Figure 3: Effect of different treatments for 4 weeks on aortic nitrite levels.

Aortic nitrite levels in different groups. All the values are represented as mean $\pm$ SEM; n=6 (p<0.05; ** vs NC; \$ vs DC; @ vs DC+Dize; # vs DC+TDC.

Figure 4: Light microscopic pictures illustrating the Picrosirius red (PSR) staining for collagen deposition (400 $\times$ magnification) and quantification of (B) % positive area of collagen.

All values are represented as means $\pm$ SEM; n=6 (p<0.05; ** vs NC; \$ vs DC; @ vs DC+Dize; # vs DC+TDC.

Table-1: pD2 (-Log EC50) values of acetylcholine and maximal response to each treatment (Emax). (p<0.05; ** vs NC; \$ vs DC; @ vs DC+Dize; # vs DC+TDC).

Groups	Acetylcholine	
	pD2	Emax (% relaxation)
NC	7.25 ± 0.01	86.93 ± 3.14
DC	6.81 ± 0.02	42.51 ± 2.17*
DC+Dize	6.96 ± 0.12	63.22 ± 6.32\$
DC+TDC	6.91 ± 0.21	61.74 ± 4.02\$
DC+Combination	7.02 ± 0.12	80.33 ± 5.22\$@#

Figure. 1

Figure 1: Effect of different treatments on plasma glucose levels and aortic oxidative stress parameters. All the values are represented as mean $\pm$ SEM; $n=6$ ($p<0.05$; * vs NC; \$ vs DC; @ vs DC+Dize; # vs DC+TDC. (a) Plasma glucose levels (b) Reduced GSH levels (c) MDA levels post treatment. NC: Normal Control; DC: Diabetic control; DC+Dize: Diabetic control treated with diminazene aceturate; DC+TDC: Diabetic control treated with tauroursodeoxycholic acid; DC+ Comb.: Diabetic control treated with a combination of diminazene aceturate and tauroursodeoxycholic acid.

338x190mm (300 x 300 DPI)

Figure 2: Concentration dependent relaxation of acetylcholine on aortic rings (with intact endothelium) pre-contracted with PE obtained from different treatment groups. Tension is expressed as % relaxation on initial contraction with PE. Values are expressed as mean $\pm$ SEM. ($p < 0.05$; * vs NC; \$ vs DC; @ vs DC+Dize; # vs DC+TDC).

338x190mm (300 x 300 DPI)

Figure 3: Effect of different treatments for 4 weeks on aortic nitrite levels. Aortic nitrite levels in different groups. All the values are represented as mean $\pm$ SEM; n=6 ($p<0.05$; ** vs NC; \$ vs DC; @ vs DC+Dize; # vs DC+TDC).

338x190mm (300 x 300 DPI)

Figure 4: Light microscopic pictures illustrating the Picrosirius red (PSR) staining for collagen deposition (400× magnification) and quantification of (B) % positive area of collagen. All values are represented as means $\pm$ SEM; $n=6$ ($p<0.05$; ** vs NC; \$ vs DC; @ vs DC+Dize; # vs DC+TDC).

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