Understanding the Role and Regulation of Endoplasmic Reticulum Stress in the Development of Endothelial Dysfunction and Kidney Disease under Type 1 Diabetic Condition

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CERTIFICATE

This is to certify that the thesis entitled **"Understanding the Role and Regulation of Endoplasmic Reticulum Stress in the Development of Endothelial Dysfunction and Kidney Disease under Type 1 Diabetic Condition**" and submitted by **Mr. Himanshu Sankrityayan,** ID No**. 2018PHXF0036P** for the award of Ph.D. of the institute embodies original work done by him under my supervision.

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Himanshu Sankrityayan

List of abbreviations

ACE	Angiotensin-converting enzyme
ACE2	Angiotensin-converting enzyme 2
ACEi	Angiotensin-converting enzyme inhibitor
AGT	Angiotensinogen
AKI	Acute kidney injury
Ang II	Angiotensin II
Ang (1-7)	Angiotensin 1-7
ANOVA	One-way analysis of variance
ARB	Ang II receptor blockers
AT1R	Angiotensin II type 1 receptor
AT2R	Angiotensin II type 2 receptor
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
BiP	Binding immunoglobulin protein
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
cGMP	Cyclic guanosine monophosphate
ChIP	Chromatin-immunoprecipitation
CHOP	CCAAT-enhancer-binding protein homologous protein
CKD	Chronic kidney diseases
Cypro	Cyproheptadine
CVDs	Cardiovascular diseases
DC	Diabetic control
DKD	Diabetic kidney disease
DM	Diabetic Mellitus
DMEM	Dulbecco's modified eagle's medium
Dize	Diminazene Aceturate
ECM	Extracellular matrix
Eif2α	Eukaryotic initiation factor 2a
ER	Endoplasmic reticulum
ERS	Endoplasmic reticulum stress
ESRD	End-stage renal disease

ET-1	Endothelin-1
EZH2	Enhancer of Zeste Homolog 2
GRP78	Glucose-regulated protein 78
GSH	Reduced glutathione
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
HIF-1α	Hypoxia-inducible factor-1α
HMT	Histone methyltransferases
HUVEC	Human umbilical vein endothelial cells
I/R	Ischemia/reperfusion
IRE1α	Inositol-requiring enzyme 1 α
IRI	Ischemic renal injury
LSB	Low salt buffer
MCP-1	Monocyte chemoattractant protein 1
MDA	Malondialdehyde
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
NC	Normal control
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor 1
c-PARP	cleaved-Poly-(ADP-ribose) polymerase
4-PBA	4- Phenyl butyric acid
PCr	Plasma creatinine
PERK	Protein kinase R-like endoplasmic reticulum kinase
PGL	Plasma glucose
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PSR	Picrosirius Red
PTMs	Posttranslational modifications
qRT-PCR	Quantitative real-time polymerase chain reaction
RAS	Renin-angiotensin system
rhACE2	Recombinant human ACE2
ROS	Reactive oxygen species
RTN1a	Reticuloin 1a
SET7/9	SET domain–containing lysine methyltransferase 7/9

STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
TGF-β	Transforming growth factor-β
TLR4	Toll-like receptor 4
TUDCA	Tauroursodeoxycholic acid
TNF-α	Tumor necrosis factor-α
UUO	Unilateral ureteral obstruction
UPR	Unfolded protein response
VPA	Valproic acid
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VSMC	Vascular Smooth Muscle Cell
WHO	World Health Organization
XBP1	X-Box binding protein 1
XTT	sodium 3´-[1- (phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy6-nitro) benzene sulfonic acid hydrate

Abstract

Persistent hyperglycemia in type 1 diabetes triggers numerous signaling pathways which leads to different complications such as diabetic kidney disease (DKD), endothelial dysfunction, retinopathy etc. Diabetes and associated complications are diseases of multiple etiology and difficult to control targeting a single pathway. This study was conceived to evaluate the potential of two different combinations using ER stress inhibitor in combination with angiotensin converting enzyme 2 (ACE2) activator and angiotensin II type 1 receptor blocker (AT1R blocker) against two different complications of diabetes, endothelial function and DKD respectively. This study also delves into the understanding the regulation of ER stress during the development of DKD using epigenetic tools.

Since, hyperglycemia damages endothelial layer via multiple signaling pathways including enhanced oxidative stress, downregulation of angiotensin converting enzyme 2 signaling and exacerbation of endoplasmic reticulum stress etc., hence it becomes difficult to prevent the injury using monotherapy. Thus, present study was aimed 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 precontracted rings. Combination therapy significantly improved the 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.

Persistent hyperglycemia causes metabolic perturbations such as mitochondrial dysfunction, oxidative stress, and endoplasmic reticulum (ER) stress leading to inflammation and, ultimately, renal fibrosis, which is the last and common pathway in the etiology of DKD. Targeting the usual glycemic, hemodynamic, and metabolic pathways with the available therapeutics have not prevented the progression of DKD suggesting an epigenetic involvement. The histone methyltransferase SET7/9 besides the methylation of histones are also known to methylate non-histone proteins, and thus its inhibitors could be useful as a therapeutic candidate. SET7/9 has specifically been linked to fibrosis, renal and extra-renal previously.

SET7/9 and ER stress could be interlinked and simultaneously involved in advancing the DKD. This study aimed to explore the role of SET7/9 in ameliorating the hyperglycemia-induced inflammation and fibrosis in kidney cells and whether it also involves the amelioration of the ER stress using a novel SET7/9 inhibitor Cyproheptadine. Our study revealed that hyperglycemia leads to increased histone H3 lysine mono- and di-methylation (H3K4Me1/2) along with elevated levels of SET7/9 and ER stress, inflammation and fibrosis. Cyproheptadine not only reduced the histone H3 lysine (K4) mono-methylation but also significantly reduced the ER stress, inflammation and fibrosis.

To study the effect of TUDCA in combination with Telmisartan we employed both *in-vitro* and *in-vivo* approaches where NRK-52E cells were incubated with high glucose, and DKD was induced in Wistar rats using streptozotocin (55 mg/kg). After 4 weeks, animals were administered with TUDCA (250 mg/kg), telmisartan (10 mg/kg), and their combination for 4 weeks. Lastly, plasma was collected for the biochemical estimation, and the kidneys were used for immunoblotting, PCR, and histopathological analysis. Similarly, for *in-vitro* experiments, cells were exposed to 1000 µM of TUDCA and 10 µM of telmisartan, and their combination, followed by cell lysate collection and immunoblotting analysis. We observed that the combination therapy was more effective in restoring the renal function decline and suppressing the apoptotic and fibrotic signaling than monotherapies of AT1R blocker and ER stress inhibitor.

In conclusion, we found that the combination therapy of TUDCA with diminazene aceturate and Telmisartan proved to be better than the monotherapies in preventing endothelial dysfunction and kidney disease in Type 1 diabetic condition. Since TUDCA is approved for clinical usage and available as a nutraceutical over the counters, thus concomitant administration of it with conventional therapies could be a novel therapeutic strategy against diabetic complications. Further, we saw that SET7/9 is vital to progression of DKD and cyproheptadine could be used as a novel inhibitor of SET7/9 in the prevention of DKD progression.

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Chapter 1

Introduction

1. Introduction

Diabetes is branded as an epidemic of the current century and, along with its complications, poses substantial health and economic burden globally. The disease diabetes mellitus, or diabetes, is among the most complex in human history. This group of metabolic disorders is characterized by prolonged hyperglycemia due to a defect in insulin secretion or action. Hyperglycaemia associated with chronic diabetes can damage, malfunction, and fail various organs, including the eyes, kidneys, nerves, hearts, and blood vessels (Mustapha et al., 2021). According to the World Health Organization, there were 451 million cases of adult diabetes in 2017, and the number is forecast to increase to 693 million by 2045 (Cho et al., 2018). China and India, for example, have significantly rising diabetes rates (Zheng et al., 2018).

Primarily strict glycaemic control and therapies targeting the metabolic, inflammatory, and hemodynamic pathways have remained the backbone of diabetic and complications treatment. However, despite several years of clinical use and research, the currently available therapies to avert the progression of diabetes and associated complications have not been entirely successful necessitating the revelation and targeting of novel molecular pathways. The different molecular mechanisms which are involved in diabetic complications include renin-angiotensin system (RAS) signaling pathways, oxidative stress, autophagy, epigenetic pathways, mitochondrial stress, and endoplasmic reticulum (ER) stress, etc (Lin et al., 2018; Sakashita et al., 2021). All these pathways are under immense scrutiny for the development of effective therapy.

ER stress is emerging as a major signaling pathway involved in the development and progression of Diabetes and associated complications including vascular endothelial dysfunction (Cunard, 2015; Maamoun et al., 2019a). Diabetes is associated with persistent hyperglycemia and insulin resistance requiring excessive demands on insulin production. The enhanced metabolic demand to produce insulin associated with diabetes leads to the generation of unfolded and misfolded proteins ultimately causing ER stress (Kataoka and Noguchi, 2013). To mitigate the persistent ER stress, an unfolded protein response (UPR) is initiated which primarily works via the following 3 signaling pathways, protein kinase R/PKR-like ER kinase (PERK), inositol-requiring enzyme 1a (IRE1a), and activating transcription factor 6 (ATF6) as described in earlier reports (Mohammed-Ali et al., 2017; Pandey et al., 2019; Xie et al., 2021). Together, these work to either halt the global protein synthesis or enhance the transcription of molecular chaperones, thus reducing the protein load. Moreover, studies also suggest that

dysregulated RAS signaling during diabetes including increased angiotensin-II or decreased angiotensin-(1-7) also elevates the ER stress and activation of the UPR in DKD and vascular endothelial dysfunction (Gomolak and Didion, 2014; Papinska and Rodgers, 2018; Wang, J. et al., 2015). Therapies targeting the dysregulated RAS including angiotensin II type 1 (AT1R) receptor blockers are already used clinically for diabetic complications. In the past few years, ER stress inhibitors have emerged as an attractive therapeutic strategy against different diseases including DKD and endothelial dysfunction (Amodio et al., 2018; Kusaczuk, 2019; Ma et al., 2021; Singh et al., 2021; Zhou et al., 2021).

Tauroursodeoxycholic acid (TUDCA) is the taurine conjugate of the bile acid largely found in the bile of the bear. The unconjugated form, ursodeoxycholic acid is already approved by US-FDA for the treatment of primary biliary cholangitis (Kusaczuk, 2019). It has been used for centuries in traditional Chinese medicine for liver and biliary disorders. However, in the past few decades, the pleiotropic property of TUDCA has emerged. It has proven to be effective in a battery of diseases such as neurological disorders, diabetic retinopathy, nephropathy, cancer, etc (Gaspar et al., 2013; Marquardt et al., 2017; Park et al., 2016; Zangerolamo et al., 2021). Initially, the protective activities of TUDCA were attributed to its chaperoning potential due to which it reduced the endoplasmic reticulum (ER) stress. However, now several other mechanisms are proposed such as anti-oxidative, anti-apoptotic, and anti-inflammatory for the cytoprotective activity of TUDCA (Kusaczuk, 2019). Currently, it is also under clinical trial for new-onset type 1 diabetes mellitus (T1DM) and endothelial dysfunction related to type 2 diabetes mellitus (T2DM) (Robin Goland et al., 2019).

The macrovascular complications are the major contributors to the morbidity and mortality associated with diabetes (Bjornstad et al., 2018; Varma et al., 2005). This could be primarily attributed to the endothelial dysfunction, which results due to the elevated oxidative stress and ER stress owing to the persistent hyperglycemia (Maamoun et al., 2019b; Rajendran et al., 2013). Moreover, endothelial dysfunction is considered an important target to prevent the progression of cardiovascular disorders (Versari et al., 2009). The hallmark and primary reason behind endothelial dysfunction development is the reduced bioavailability of nitric oxide (NO). Several pathophysiological conditions may reduce the NO levels, hyperglycemia being one of them (Chen et al., 2018). Also, persistent hyperglycemia is well known to alter the pressor arm of the renin-angiotensin system and increase the levels of angiotensin-II, which in turn increases the oxidative stress (Lavrentyev et al., 2007; Pueyo et al., 2000) and ER stress in endothelial cells (Liang et al., 2013; Murugan et al., 2015). ER stress could suppress the eNOS expression, thus reducing the NO level in the aortic rings obtained from mice (Lau, Y. S. et al.,

2018). Studies even suggest a probable mechanistic link between ER stress and oxidative stress being implicated in cardiovascular disorders associated with diabetes, probably due to both being important mediators of endothelial dysfunction (Ochoa et al., 2018). Furthermore, hyperglycemia is also known to reduce the angiotensin-converting enzyme- 2 (ACE2) levels which forms the depressor arm of the renin-angiotensin system and is vasoprotective in nature. ACE2 knockout animals had vascular dysfunction due to reduce NO bioavailability and enhanced oxidative stress (Rabelo et al., 2016). ACE2 and angiotensin (1-7) are emerging as an important therapeutic target to counter the endothelial dysfunction. Direct administration of angiotensin (1-7) 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, current study employed simultaneous inhibition of endoplasmic stress and activation of ACE2 by novel combination of diminazene aceturate (ACE2 activator) and TUDCA (ER stress inhibitor) respectively for better vasoprotection.

Diabetic kidney disease (DKD) is a highly prevalent microvascular complication of diabetes and is primarily responsible for the development of end-stage renal disease (ESRD) (Fu et al., 2019). The cases of DKD are on a constant uprise which could be attributed to the epidemiological proportion increase in diabetic patients across the globe (Alicic, Radica Z. et al., 2017). Several factors are responsible for developing DKD, hyperglycemia and hypertension being the most prominent ones (Alicic, Radica Z. et al., 2017; Lin et al., 2018). Downstream to the persistent hyperglycemia lies the inflammatory pathways and the consequential activation of the profibrotic pathways leading to renal fibrosis and culminating in ESRD (Furuya et al., 2019; Kanasaki et al., 2013; Wada and Makino, 2013). Apart from strict glycemic control, renin-angiotensin system (RAS) inhibitors have been used in preventing the progression of DKD for decades, albeit with much success (Yamazaki et al., 2021). The primary characteristics of DKD include hypertrophy in the glomerulus, tubulointerstitial fibrosis, mesangial expansion etc. Moreover, injury to the tubular epithelial cells is vital to the progression of DKD. Hyperglycemic exposure acts as an initiator of tubular cell injury (Tong et al., 2018). Tubular epithelial cells are prone to the development of ER stress, which is produced due to persistent hyperglycemia (Fang et al., 2013), thus aggravating

DKD. A kidney is one of the sites with a high rate of protein synthesis and the metabolic derangements during diabetes further tend to elevate the ER stress (Fougeray et al., 2011; Tessari et al., 1996).

DKD is a disease of multiple etiologies, and in the past few decades, the role of epigenetics in regulating critical genes involved in the DKD has come to the fore (Lu et al., 2021). Epigenetics consists of a change in the expression and function of the gene without alteration in the genetic sequence and is also heritable. For instance, post-translational histone modifications (PTHMs) such as acetylation, methylation, phosphorylation, and ubiquitylation are essential in regulating chromatin structure, and hence genomic DNA accessibility (Kato and Natarajan, 2019). A set of different enzymes carries out these modifications, HMTs (Histone Methyl Transferases) for histone methylation, Histone Acetyl Transferases (HATs) and Histone Deacetylases (HDACs) etc. (Kato and Natarajan, 2019; Zheng et al., 2021). SET domain-containing lysine methyltransferase 7/9 (SET7/9) is one such enzyme that has been implicated in the development of fibrosis lately (Sasaki et al., 2016; Tamura and Doi, 2018). SET7/9 is a histone methyltransferase that primarily induces histone H3 lysine (K4) mono-methylation (H3K4Me1). Studies showed that transforming growth factor-beta 1 (TGF-β1) is involved in the aggravated expression of SET7/9, and the consequent increase in H3K4Me1 may add to the fibrotic cascade (Guo et al., 2016; Sasaki et al., 2016; Shima et al., 2017).

Hyperglycemia has also been implicated in the generation of ER stress which adds to the progression of DKD (Lindenmeyer et al., 2008a; Wang, W.-W. et al., 2021). ER stress also regulates the inflammatory cascade in diabetic conditions (Qi et al., 2011). Moreover, SET7/9 has been reported to regulate the ER stress in hepatocytes, and vice-versa has been found in DKD (Chen, J. et al., 2014; Han et al., 2021). Overall, there exists an intricate relationship between SET7/9 expression, ER stress and hyperglycemia, which further regulates the downstream inflammatory and fibrotic pathways. Recently, Cyproheptadine, a clinically approved drug, has emerged as a SET7/9 inhibitor in a few studies (Hirano et al., 2018; Takemoto et al., 2016). Takemoto et al., in their study, used a high throughput HMT assay based on fluorogenic substrates to screen for the inhibitors of SET7/9. Interestingly, amongst several compounds screened by them, they found Cyproheptadine to be an outstanding candidate for the inhibition. During confirmatory studies, it was found that Cyproheptadine inhibits SET7/9 activity with a half-maximum inhibitory concentration (IC50) value of 1.0 μM (Takemoto et al., 2016). Increased expression of SET7/9 and ER stress in renal tubular epithelial cells has proved to be a significant determinant in the development of renal fibrosis associated with DKD. Therefore, we hypothesized to evaluate the potential of Cyproheptadine

in preventing hyperglycemia-induced damage to the renal tubular epithelial cells and the underlying molecular mechanisms.

Also, diseases such as DKD which has complex etiology are relatively difficult to control using a single therapy. Hence, combining different forms of therapies such as strict hyperglycemic control with different RAS inhibitors, SGLT2 inhibitors, DPP4i are considered to be the newer approach to control the progression of DKD efficiently (Cai et al., 2020). This study aimed to evaluate the potential of TUDCA an ER stress inhibitor in combination with Telmisartan, an AT1R blocker to provide a novel and improved therapeutic strategy against DKD.

Overall, the study aims to delineate the role of ER stress in the development and progression of diabetic complications, including vascular endothelial dysfunction and DKD. Furthermore, the study will also explore the epigenetic mechanisms of the regulation of ER stress in kidney. Since, the diabetic complications are difficult to manage using single therapy and use of alternative complimentary medicine is increasing so we intend to use a novel combination of TUDCA with an ACE2 activator and AT1R blocker against endothelial dysfunction and DKD respectively. Combining the pleiotropic TUDCA with RAS modulators could prove to be a useful strategy against diabetic complications of complex origin.

Chapter 2

Review of Literature

2. Review of Literature

2.1. Diabetes and Associated Complications

Diabetes has already been branded as an epidemic of the current century and along with its complications poses substantial health and economic burden globally. Diabetic complications are majorly categorized into microvascular complications and macrovascular complications. Diabetic macrovascular complications could be defined as the effect of chronic hyperglycaemia in the pathological remodelling of the larger blood vessels. The frequently reported macrovascular complications are stroke, peripheral vascular disease, cardiovascular diseases (CVD) etc. The advent of CVD in diabetic patients has been reported ins several reports which are independent of other traditional CVD risk factors implicating indisputable link between diabetes and CVD. As per statistics, CVD is still the most frequent reasons of death in diabetic patients, with almost 52% mortality rate in T2DM patients and 44% deaths in T1DM patients (Huang et al., 2017). The increase in CVD and related deaths during diabetes has vascular endothelial dysfunction as a primary culprit. As per a recent metanalysis including 58 independent reports projects a very early presence of impaired endothelial function in T1DM patients. Not only this, studies suggest that more than 35% of individuals develop endothelial dysfunction in a span of 5 years of being diagnosed with T1DM.

Diabetic microvascular complications can be defined as the sequels of diabetes due to persistent and inadequately controlled hyperglycaemia primarily affecting the smaller blood vessels and capillaries which includes nephropathy, retinopathy, and neuropathy. Interestingly, the time period between detection of diabetes and development of microvascular complications is much smaller as compared to that in the development of macrovascular complications (Seid et al., 2021). Also, microvascular complications are more prevalent with almost 50% of the people with T2DM across 28 countries of Europe, South America, Asia, and Africa found to be afflicted with it (Zheng et al., 2018). The a1chieve study, an Indian observational study encompassing 20,554 T2DM patients, reported the prevalence of nephropathy, retinopathy, and neuropathy to be 30.2%, 32.5%, 26.8% respectively (Mohan et al., 2013). Moreover, in a retrospective cohort study performed in 135 199 T2DM patients the prevalence of diabetes associated CKD was highest (12.3%) at the time of diagnosis (An et al., 2021).

2.2. ER Stress and Diabetes

Multitude of studies points at an active role of pancreatic β cell ERS in the pathophysiology of diabetes (Clark and Urano, 2016; Evans-Molina et al., 2013). Although T1DM and T2DM have different etiology and different triggers, malfunctioning and longevity of β cells are common to both T1DM as well as T2DM (Eizirik et al., 2008). T2DM is characterized by persistent hyperglycemia because of the inability of β cells to synthesize insulin or resistance of different tissues towards insulin (Back and Kaufman, 2012). ER is the site for the synthesis, processing, and storage of proinsulin, a precursor of insulin (Sun et al., 2015). Insulin is released in the circulation as per the requirement. According to literature elevated blood glucose may lead to a steep rise in translation rate of proinsulin synthesis which may be as high as ~20 fold, and almost a million proinsulin molecules are formed in a minute (Back and Kaufman, 2012). Even in normal physiological conditions, almost $1/5^{th}$ of the proinsulin synthesized doesn't attain its native conformation showing that proinsulin is highly susceptible to misfolding. The high biosynthetic burden on pancreatic β cells compounded with misfolding prone nature of proinsulin puts the β cell's ER under ERS (Sun et al., 2015). This activates the UPR signaling pathways to remove the misfolded proinsulin molecules. However, due to persistent hyperglycemia and chronic ERS associated with diabetes, the protective UPR pathways turns destructive for the β cells. Studies support the claim that ERS induced by chronic hyperglycemia and hyperlipidemia leads to loss of β cells mass and function in T2DM (Salvado et al., 2015). Amongst the three UPR sensors PERK is reported to play an important role in the regulation of β cell development, proliferation, homeostasis along with a prominent role in insulin processing and secretion (Kefalas and Larose, 2018). This is corroborated by studies which showed that PERK-deficient mice exhibited severe β cell malfunctioning and diabetes. Gao at al. found in their study that PERK deletion in young as well as adult mice led to structural damage and loss of islet and the β cells resulting in high glucose levels (Gao et al., 2012). In a recent finding by Yimeng et al. abrogation of PERK- CCAAT-enhancer-binding protein homologous protein (CHOP) pathway using sodium butyrate led to amelioration of T2DM (Hu et al., 2018). PERK was also involved in controlling the proinsulin processing by regulating the expression of ER chaperones (Sowers et al., 2018). PERK was first found in islets of rat pancreas and play an important role in Wolcott–Rallison syndrome (WRS), a rare genetic disorder typified by early onset/neonatal diabetes with a reduction in the mass of β cell not related to autoimmune destruction (Delepine et al., 2000). In physiological circumstances IRE1α aids to enhanced proinsulin biosynthesis after a meal. However, persistent exposure to high glucose leads to ERS induced overactivation of the IRE1α signaling pathway which

reduces the expression of insulin gene in β cells (Lipson et al., 2006). Tsuchiya et al. in a recent report explored the IRE1α - X-box binding protein 1 (XBP1) pathway in maintaining the proinsulin and insulin levels along with oxidative folding of the proinsulin molecules (Tsuchiya et al., 2018). They found that deficiency of IRE1α in mouse β cells led to the development of diabetes with reduced biosynthesis, secretion, and folding of insulin and proinsulin (Tsuchiya et al., 2018). The β cell homeostasis depends on the balance between the activity of PERK and IRE1α since PERK regulate insulin biosynthesis negatively wheras IRE1α regulate it positively (Corbett, 2006). Any imbalance between the two may be detrimental to the weathering of β cells. The role of ATF6, the third component of UPR, in diabetes is limited, which warrant further investigation in the development of diabetes.

ER Stress is also known to be actively involved in the development of T1DM, an autoimmune disorder characterized with destruction of pancreatic β cells (Engin et al., 2013). Earlier it was found that β cell dysfunction is antecedent to T1DM and ER Stress is a causative factor for the same which can be attributed to activation of NF-κB pathway (Eizirik et al., 2008). Impairment in the UPR components including ATF6 and XBP1 in the β cells of T1DM mouse model and human samples exemplify the role of ERS and UPR in maintenance of β cells and prevention of T1DM (Engin et al., 2013). In addition, presence of ERS and activation of UPR mediators in the pancreatic islets was observed in 13 T1DM patients (Marhfour et al., 2012). ERS was found to precede insulitis much earlier forming microenvironment conducive to development of autoimmunity and T1DM (Crookshank et al., 2018). It was demonstrated that β cell proteins become immunogenic via Ca^{2+} -regulated post-translational modification when exposed to thapsigargin and physiological triggers to ERS (Marre et al., 2016). ERS is one of the hallmarks of T1DM in humans and elevated levels of pro-inflammatory cytokines interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNFα) and interferon- alpha (IFNα) leads to elevation of ER stress adding to the progression of T1DM (Engin, 2016). Islet cells isolated from 12 non-diabetic donors were exposed to different cytokines including IFNα and IL-1β, which interestingly caused a 2-fold increase in β cell apoptosis within 24 hours (Marroqui et al., 2017). Treatment with TUDCA or knockout of CHOP in these cells led to reduced apoptosis indicating the involvement of ERS in IFNα and IL-1β mediated apoptosis (Marroqui et al., 2017). Thus, targeting ERS and UPR components holds tremendous potential as a novel approach to target diabetes.

2.3. ER Stress and UPR

Protein is the structural and functional unit of a cell. Its biological activity depends on the three-dimensional conformational structure that it acquires after synthesis by ribosomes in the ER (Stolz and Wolf, 2010). The ER is a voluminous membrane-bound organelle involved in several physiologically vital functions e.g. synthesis, folding, processing, and transport of proteins, synthesis of lipids, storage and release of Ca^{2+} and in signaling operations (English and Voeltz, 2013; Phillips and Voeltz, 2016). The process of protein synthesis to folding and transport is highly complex and is prone to errors. Improper protein folding may lead to several pathophysiological conditions (Menzies et al., 2011; Wang and Kaufman, 2016). Interestingly, almost one-third of the proteins synthesized by ER in normal physiological conditions are misfolded suggesting that the situation can be much worse during pathological states of the cell (Romero and Summer, 2017). However, ER maintains the homeostasis between protein manufacture, folding, transport and degradation, a process termed as proteostasis (Inagi et al., 2014; Wang and Kaufman, 2016). An intricate set of mechanisms continually monitor the quality and quantity of the proteins synthesized, e.g. molecular chaperones assist in proper folding of the proteins as well as degradation of misfolded proteins (Almanza et al., 2018). Nevertheless, different environmental and genetic cues along with metabolic alterations may disturb the normal course of protein synthesis and folding leading to activation of an ubiquitin-proteasome system and macroautophagy and ERS (Salvado et al., 2015). The primary response to ERS is activation of UPR which acts as a sensor looking over the workload of ER and acts via different mechanisms to mitigate the accumulated misfolded proteins (Lindholm et al., 2017). UPR alleviates ERS broadly by following ways: reduction in the load on ER via global inhibition of synthesis of new proteins, enhanced transcription of constituents of ERAD which aids in degradation of misfolded proteins and by enhancing the ER activity and size to increase protein synthesis and folding capability (Herbert and Laybutt, 2016). Three major branches of UPR are reported to function in parallel by employing a distinct set of signaling pathways. These signal transducers consist of transmembrane proteins namely Protein Kinase R/PKR-like ER kinase (PERK), inositol-requiring enzyme 1α (IRE1**α**) and activating transcription factor 6 (ATF6) (Almanza et al., 2018). Under resting conditions UPR sensors are bound to BiP which keeps them in inactive state. However, when unfolded proteins aggregate the affinity of BiP for UPR sensors decreases and it separates from the sensors thus activating the UPR signaling pathway (Cybulsky, 2017). PERK reduces the protein load by inhibiting translation. PERK also acts as an important part of mitochondrial-associated ER membranes (MAMs) and maintenance of mitochondria-ER, plasma membrane-ER contact sites by interacting with

Filamin A. PERK is also reported to regulate apoptosis, inflammation and intracellular Ca^{+2} entry (van Vliet and Agostinis, 2017). IRE1**α** and ATF6 act by inducing the transcription of UPR target genes and chaperones thus facilitating the removal of accumulated unfolded proteins. IRE1**α** was also found to be an important factor in formation of MAMs which in turn regulate mitochondrial Ca^{+2} uptake (Carreras-Sureda et al., 2019). IRE1α also plays an important role in facilitating the interaction between ER and mitochondria thus maintaining cellular bioenergetics (Carreras-Sureda et al., 2019). Akin to PERK, IRE1α is also reported to directly interact with filamin A and regulate actin cytokeleton remodelling (Urra et al., 2018). ATF6, the third branch of the UPR also plays an important role in tissue development (osteogenesis, adipogenesis, neuroembryogenesis), homeostasis and pathogenesis. Villalobos-Labra et al. in their report stated that ATF6 was localized in the nucleus of Human umbilical vein endothelial cells (HUVECs) of pre-pregnancy maternal obese subjects corroborating the role of obesity in the development of ER stress (Villalobos-Labra et al., 2018). UPR is a protective mechanism, it may switch its pro-survival mode to pro-apoptotic mode on regular periods of elevated ERS (Walter and Ron, 2011). UPR pathway is designed to mitigate the acute change in the proteostasis environment (Zhuang and Forbes, 2014). However, prolonged activation of UPR in chronic conditions like diabetes or viral infections shows that ER stress cannot be reduced. Hence, to protect the system from further damage the ER elicits multiple apoptotic pathways (Back and Kaufman, 2012).

2.4. ER Stress and Diabetic Kidney Disease

Kidney synthesizes almost 42% of total body proteins similar to pancreatic β cells (Zhuang and Forbes, 2014). Diabetes further exacerbates the rate of kidney protein synthesis thus enhancing the probability of ERS and UPR activation (Cunard, 2015). Tubular cells have a high rate of protein synthesis and are a crucial target of elevated glucose levels making it prone to ERS (Zhuang and Forbes, 2014). Subsequently, ERS inhibitor TUDCA ameliorated tubulointerstitial fibrosis in addition to lowering blood glucose level and urinary albumin to creatinine ratio (Zhang et al., 2016). TUDCA apart from inhibiting ERS also stimulates Farnesoid X receptor, abundant in kidney tubular cells is known to play a protective role in DN(Marquardt et al., 2017). Suppression of ERS markers viz binding immunoglobulin protein/ glucose-regulated proteins78 (BiP/GRP78), ATF-4, p-PERK, and CHOP also prevented tubular cell apoptosis in DN (Ju et al., 2019). Podocytes are the kidney cells crucial in the development of DN (Zhuang and Forbes, 2014). Both advanced glycation end products (AGE) and albumin induce ERS and podocyte injury (Chen et al., 2008; Gonçalves et al., 2018). ERS

reduction using TUDCA and 4-PBA protected podocytes from apoptosis in DN (Cao, A.L. et al., 2016). Chrysin alleviated podocyte injury via downregulating the PERK-eIF2α-ATF4-CHOP expression, a major ERS pathway (Kang et al., 2017). Reticulon 1 (RTN1) protein has been implicated in the progression and severity of DN since it induces ERS (Fan et al., 2015; Fan et al., 2017).

ERS and autophagy interplay in the renal tubules was first reported by Kawakami et al. using immortalized rat proximal tubular cell line (IRPTC) (Kawakami et al., 2009). ERS inducers, Tunicamycin, and brefeldin A upon exposure led to the activation of the autophagic process (Kawakami et al., 2009). Fang et al. found an interplay between ERS and autophagy in podocytes. Exposure of 3-MA led to enhanced ERS in the podocytes whereas knockout of CHOP restored the autophagy. It suggests that autophagy aided in removing the unfolded proteins thus relieving the ER from excess burden and inhibition of autophagy led to the accumulation of such proteins elevating the ERS. AGEs were found to trigger autophagy in the glomerular mesangial cells (GMCs) by ERS initiation. Autophagy modulation and ERS suppression was effectively mediated by 4-PBA. Although 4-PBA failed to modulate rapamycin-induced autophagy in GMCs indicating AGE-induced autophagy was mediated via eIF2α/CHOP stress pathway (Chiang et al., 2016). Role of ERS and autophagy has been summarized in Fig. 1.

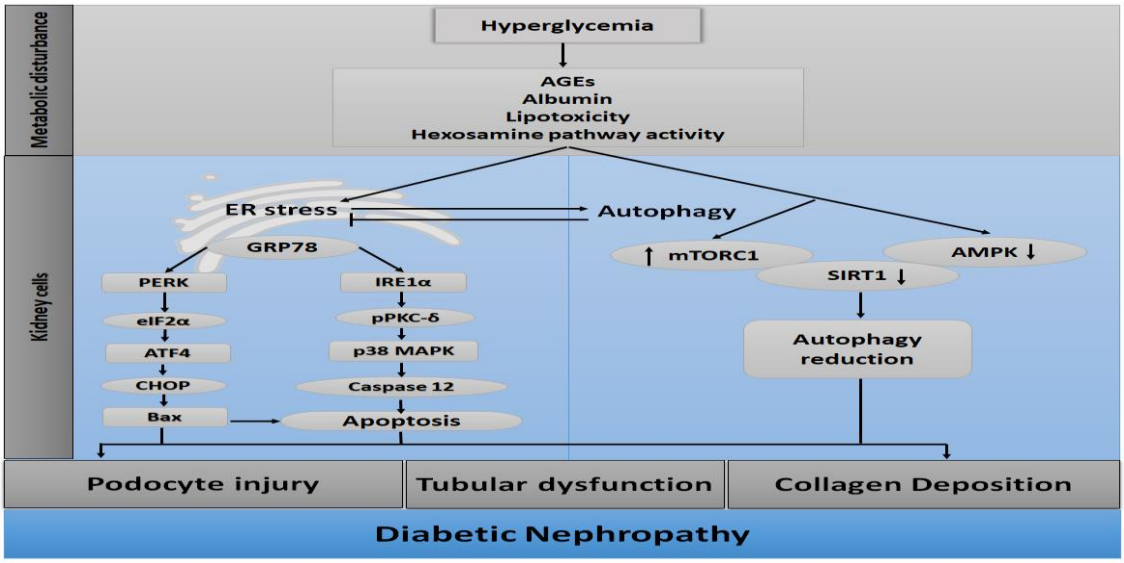


Fig. 1: A schematic representation of pathways beginning at hyperglycemia and leading to diabetic nephropathy via ER stress and autophagy: The figure depicts that hyperglycemia leads to elevated ER stress and reduced autophagy levels. This further leads to injury to podocytes and mesangial cells along with tubular cell apoptosis finally leading to diabetic nephropathy.

2.5. ER Stress and Diabetic Retinopathy

Diabetic retinopathy (DR) is described by basement membrane thickening, microaneurysms, pericyte loss, blood-retinal barrier breakdown, acellular capillary, intraretinal microvascular abnormality, neovascularization and finally retinal detachment (Cai and Boulton, 2002). Hyperglycemia allied with reactive oxygen species (ROS), inflammation and ERS appear as a censorious factor causing damage to the retinal blood vessels as well as neurons (Volpe et al., 2018). Damage to the blood-retinal barrier is an imperative feature of DR (Wong et al., 2016). Muller cells a type of critical glial cells in the retina are the key cause of inflammation in retinopathy due to its ability to secrete cytokines and various growth factors such as VEGF and ICAM-1 (Zhong et al., 2012). Overproduction of VEGF unsettles the blood-retina barrier leading to vascular exudation and neovascularization (Wong et al., 2016). Impairment in the balance of pro-angiogenic factor like vascular endothelial growth factor (VEGF) and intercellular adhesion molecule-1 (ICAM-1) and pro-inflammatory cytokines viz. TNF-α, IL-6, IL-8 and monocyte chemoattractant protein 1 (MCP1) are critical to development of DR (Wong et al., 2016; Yang et al., 2019). Yang et al., recently studied the role of XBP1, a transcription factor and an important component of the UPR in Müller cells of diabetic mice and primary Müller cells being exposed to high glucose and hypoxic conditions (Yang et al., 2019). XBP1 deficient diabetic mice had elevated levels of retinal VEGF, TNF-α and ERS markers including ATF-4, ATF-6, CHOP, GRP-78, eif2α and these mice showed higher vascular leakage (Yang et al., 2019). Similarly, primary Müller cells devoid of XBP1 witnessed higher ERS and inflammatory markers, which was attenuated by treatment with ERS inhibitors (Yang et al., 2019). Impairment in the retinal Müller cell proteins O-GlcNAcylation is another factor involved in the pathogenesis of DR. To evaluate the role of high fat diet (HFD) induced ERS O-GlcNAcylation Dai and colleagues utilized animal model as well as rat retinal Müller *cell* line (TR-MUL cells). Mice fed with HFD for four weeks displayed enhanced O-GlcNAcylation, which was attributed to elevated ERS (DAI et al., 2018). ERS was proposed to enhance the Muller cell protein O-GlcNAcylation by upregulating the expression of glutamine-fructose-6-phosphate amidotransferase 2 (GFAT2) enzyme, which in turn increases the flux from hexosamine biosynthesis pathway (HBP) (DAI et al., 2018). ERS induction using thapsigargin and inhibition using chemical chaperones enhanced and repressed the O-GlcNAcylation alongwith GFAT2 levels in TR-MUL cells corroborating the in vivo finding regarding the role of ERS in retinal protein O-GlcNAcylation and progression of DR (DAI et al., 2018). ATF4, one of the important mediators of UPR regulated the expression of VEGF and ICAM-1, pro-inflammatory cytokines vital to development of DR (Wang et al., 2017). In

a recent clinical study aqueous humor and vitreous of peripheral DR patients were evaluated and a significantly higher ATF4 levels were reported (Wang et al., 2017). The samples also had elevated levels of pro-inflammatory mediators like IL-6 and MCP-1. A correlation analysis between ERS mediators and inflammatory cytokines revealed a significant tally between ATF4 and IL-6/MCP-1 levels (Wang et al., 2017). AGE may also add on to the development of DR by different pathways including ERS. Recently, De-Wei et al observed enhancement in the vascular permeability and leakage in Nε-(carboxymethyl) lysine (CML), an AGE product, treated animals which was attributed to TPL2 (tumor progression locus 2)/ATF4/SDF1α (stromal cell–derived factor-α) axis (Lai et al., 2017). They found that ATF4 regulated the SDF1α expression, which was corroborated in human as well animal serum and aqueous humor samples (Lai et al., 2017). Elevated levels of ER stress mediators in RPE cells co-incubated with AGE showed the role of AGE-RAGE in the generation of ERS and consequent damage to the retina in diabetic mice (Kang et al., 2018). Furthermore, methylglyoxal led to enhanced apoptosis of adult human retinal pigmental epithelial cell lines (ARPE-19 cells) via ERS mediated ROS generation and intracellular calcium imbalance which was reversed by treatment with salubrinal and 4-PBA (Chan et al., 2016). GRP78 regulate ERS response via activation of UPR sensors (Shao et al., 2017). Elevated levels of GRP78 were correlated with apoptosis of hRECs and generation of inflammatory mediators in eyes of patients with DR (Shao et al., 2017). Pro-inflammatory cytokines persistent exposure in DR is an important factor in neovascularization. Corroborating the role of GRP78 in vascular angiogenesis, transthyretin (TTR) suppressed the process by elevating GRP78 and facilitating apoptosis of human retinal microvascular endothelial cells (hRECs) through eif2α/CHOP pathway (Shao et al., 2017). ERS role in the progression of DR has been summarized in Fig. 2.

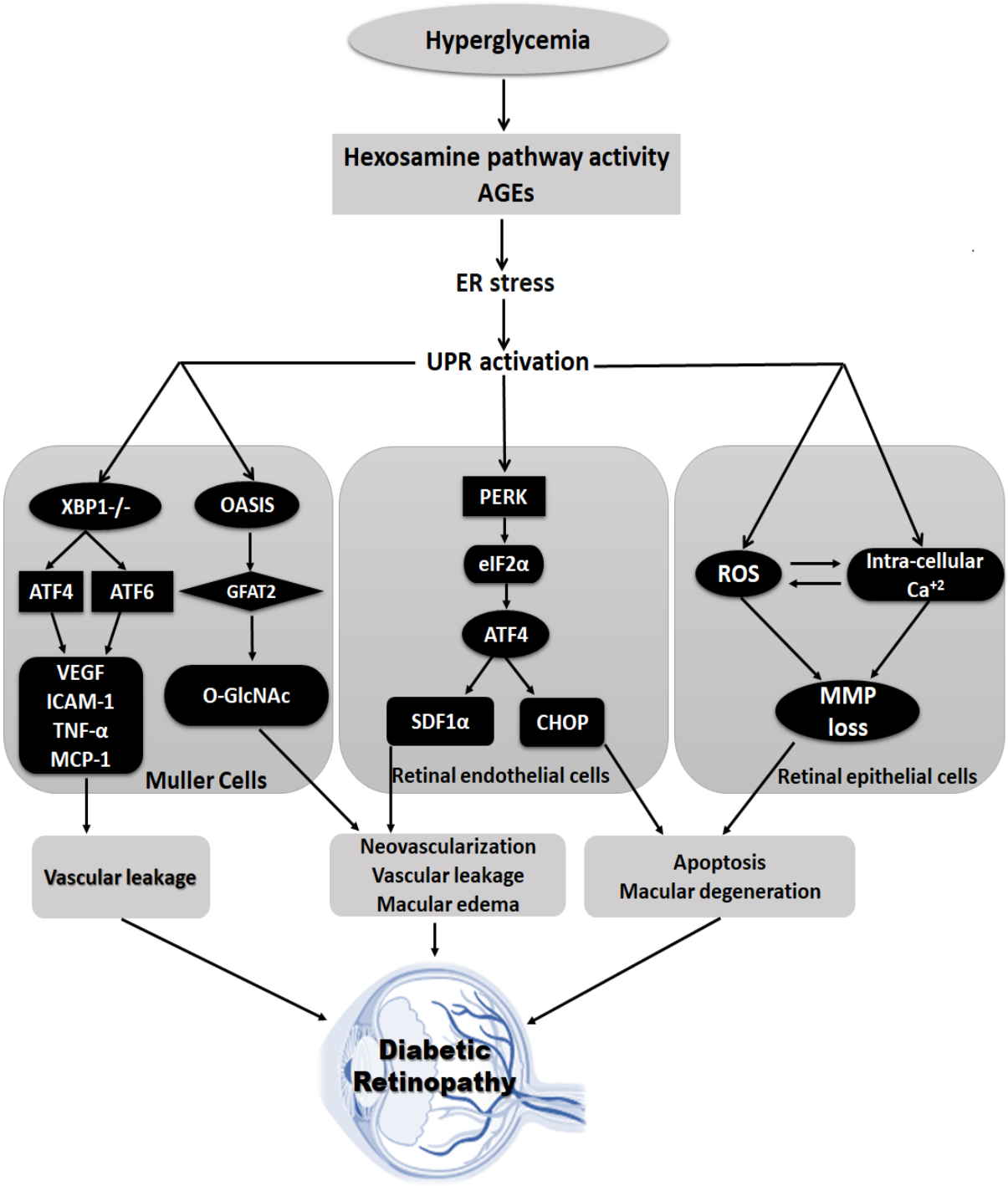


Fig. 2: A schematic representation of pathways beginning at hyperglycemia and leading to diabetic retinopathy

The figure shows elevation in ER stress mediators leading to increased vascular cell permeability, vascular leakage, retinal cell apoptosiss, generation of pro-inflammatory markers further adding to the progression of diabetic retinopathy. **AGE:** Advanced Glycation End products, **PKC:** Protein kinase C

2.6. ER Stress and Diabetic Neuropathy

Diabetic neuropathies range from acute to chronic and focal to diffused, however, diabetic peripheral neuropathy (DPN) is the most common. Diabetic neuropathy is characterized by increasing pain sensation to tactile stimulation and sensory loss to heat along with paresthesia, hyperalgesia, and allodynia (Rajchgot et al., 2019). The histopathological changes include degeneration of peripheral fibers, Schwann cell atrophy, axonal swelling and demyelination of nerve fiber (Sango et al., 2017). Hyperglycemia-induced activation of various pathway including increased activity of aldose reductase, formation of AGE, activation of protein kinase C, generation of ROS, mitochondrial dysfunction, low grade inflammation, reduced blood flow to nerves are some of the pathogenetic mechanism associated with diabetic neuropathy (Grisold et al., 2017). Inceoglu et al revealed the role of ERS and soluble epoxide hydrolase (sEH) regulation of diabetic neuropathy and associated pain. UPR sensors including PERK, IRE1α and ATF-6 along with their downstream mediators were found to be elevated in the peripheral nervous system of T1DM rats (Inceoglu et al., 2015). 1-Trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU), an oral inhibitor of sEH when administered concomitantly with 4-PBA led to a synergistic reduction in neuropathic pain concurring that ERS is one of the main culprits in the development of neuropathic pain (Inceoglu et al., 2015). Tunicamycin, a known ERS inducer results in enhanced neuropathic pain again confirming the involvement of ERS in the pathogenesis of neuropathic process (Inceoglu et al., 2015).Persistent hyperglycemia disrupts the normal functioning of the nervous system apart from morphological changes. Sharma et al. observed that incubation of dorsal root ganglion (DRG) neurons with high glucose caused increased apoptosis, which was prevented by treatment with 4-PBA suggesting the involvement of ERS in this process (Sharma et al., 2016). Schwann cells are another part of the peripheral nervous system, which is highly exposed to damage by hyperglycemia (Hao et al., 2015). Rui et al found that rats with DPN have impaired myelin sheath, nerve fibers which were attributed to excess of ERS (Li, R. et al., 2017). In vitro rat Schwann cell - RSC96 when cultured with high glucose exhibited elevated ERS as evinced by high GRP78 and CHOP levels (Li, R. et al., 2017). Furthermore, it was found that nerve growth factor (NGF) treatment both in vivo and in vitro led to significant improvement in the DPN parameters which could be ascribed to reduced ERS by NGF. (Li, R. et al., 2017). Nevertheless, the short half-life of NGF and its tendency to diffuse swiftly in physiological environment hampered its use in patients for DPN (Li, Rui et al., 2017). Rui et al. address the issue in a pre-clinical study by preparing a biodegradable coacervate of NGF and basic fibroblast growth factor (bFGF) (Li, Rui et al., 2017). They observed that a dual delivery of the

NGF and bFGF significantly alleviated DPN in vivo and in vitro RSC 96 cells (Li, Rui et al., 2017). Furthermore, incubation of Schwann cells with glycolaldehyde induced apoptosis via ERS (Sato et al., 2015). Tangluoning, a Chinese herb has been reported to alleviate ERS in DPN via PERK/Nrf2 pathway (Yang et al., 2017). In a clinical study, DPN patients showed elevated mRNA expression of CHOP indicating the potential of it in prognosis of DPN progression (El-Horany et al., 2019). Yao and Yang et al. found that IRE1α siRNA transfection inhibited pJNK, caspase 12 and CHOP expression and improved nerve morphology and demyelination in DPN rats (Yao et al., 2018). Different stimulators of ER stress leading to the development of DPN are shown in Fig. 3.

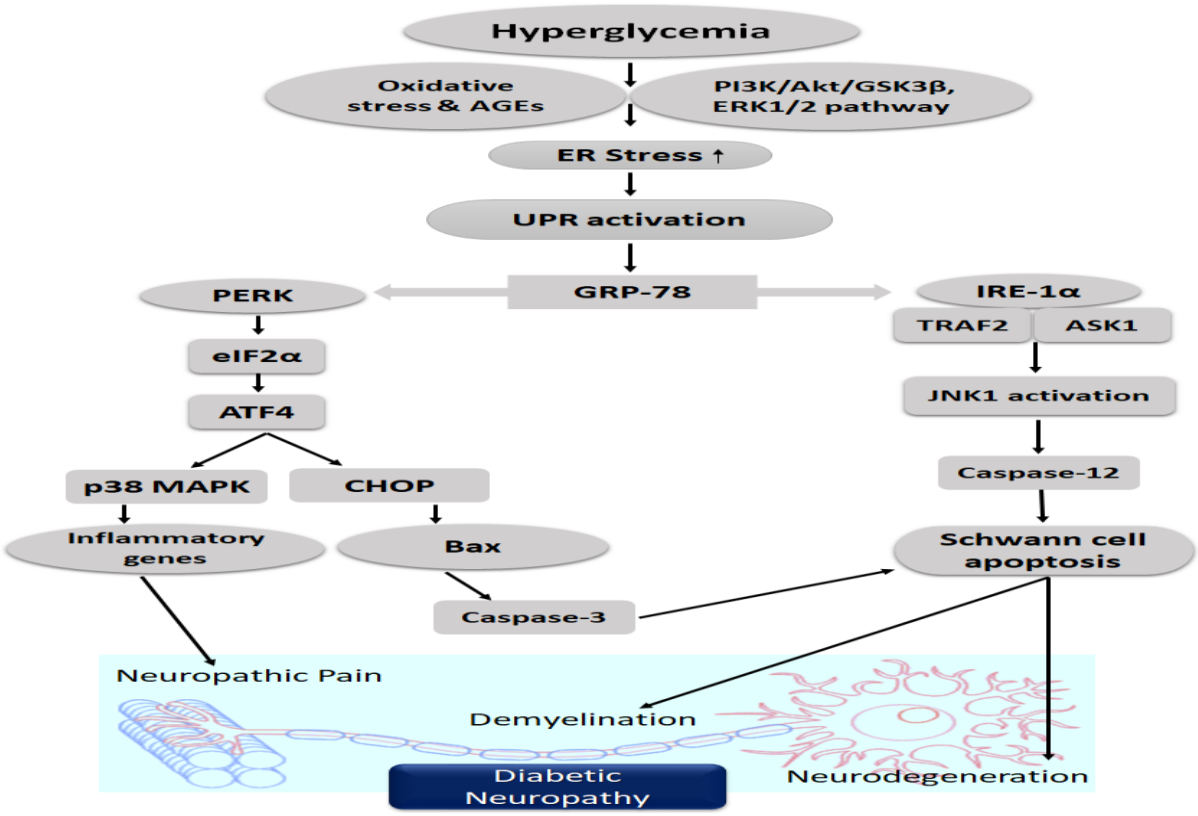


Fig. 3: A schematic representation of pathways beginning at hyperglycemia and leading to diabetic neuropathy

The figure depicts the different stimulators of ER stress including oxidative stress, mitochondrial dysfunction, inflammation and AGE accumulation leading to elevation in ER stress. This causes Schwann cell apoptosis, demyelination and other changes culminating in diabetic neuropathy. **ATF4:** Activating transcription factor 4, **ATF6:** Activating transcription factor 6, **CHOP:** CCAAT-enhancer-binding protein homologous protein, **GRP78:** Glucose regulated protein 78, **eiF2α:** Eukaryotic Initiation Factor 2α, **SDF1α:** stromal cell-derived factor α, **VEGF:** Vascular endothelial growth factor,

ICAM-1: Intercellular Adhesion Molecule 1, **IL-6:** *Interleukin 6, **MCP-1:*** Monocyte chemoattractant protein-1.

2.7. Renin Angiotensin System: Components and Role in Diabetic Complications

RAS is vital to the maintenance of kidney homeostasis. RAS is known to regulate kidney functioning and blood pressure via two prominent arms which work in exactly opposite ways. One is usually termed as the conventional arm or the pressor arm of the RAS and has been researched for decades for its role in different pathological conditions. The primary components of the pressor arm include angiotensin II/angiotensin-converting enzyme (ACE)/AT1R. The second arm is the relatively newer one and is termed as depressor arm or the non-conventional arm. It primarily consists of angiotensin-converting enzyme-2 (ACE-2)/angiotensin 1-7 (Ang 1-7)/Mas receptor (MasR). In the past few decades, both the arms of RAS have been thoroughly researched for their role in the development and progression of DKD (Fig. 4). Majority of the therapeutic options available for kidney diseases including DKD target the pressor arm of the RAS. ACE inhibitors (ramipril), and AT1R blockers (Telmisartan) are the most commonly prescribed medications for hypertension and DKD (Carey and Siragy, 2003). However, they have failed to delay or prevent the progression of DKD. This led to the enhanced exploration of the protective arm/depressor arm of the RAS. In recent years, studies exploring the role of different components of the depressor arm in several diseases including DKD, such as therapeutics altering the levels of Ang (1-7) and ACE2 have increased dramatically.

Kim et al. in their study reported that rats with unilateral ureteral obstruction (UUO), when infused with angiotensin (1-7) showed improvement in the kidney injury by reducing the fibrosis and apoptosis (Kim et al., 2015). We ourselves have extensively explored the role of the depressor arm of RAS in the development of the DKD. Goru et al. reported that activating the ACE2 using diminazene aceturate prevented the progression of DN in TID rats. Furthermore, chronic administration of Ang (1-7) has been linked to the prevention of metabolic syndrome caused by a high-fructose/low-magnesium diet (HFrD) in rats (Marcus et al., 2013). Still, a lot is left to be unearthed regarding the role of both the arms of RAS as far as the therapeutic development for DKD is concerned.

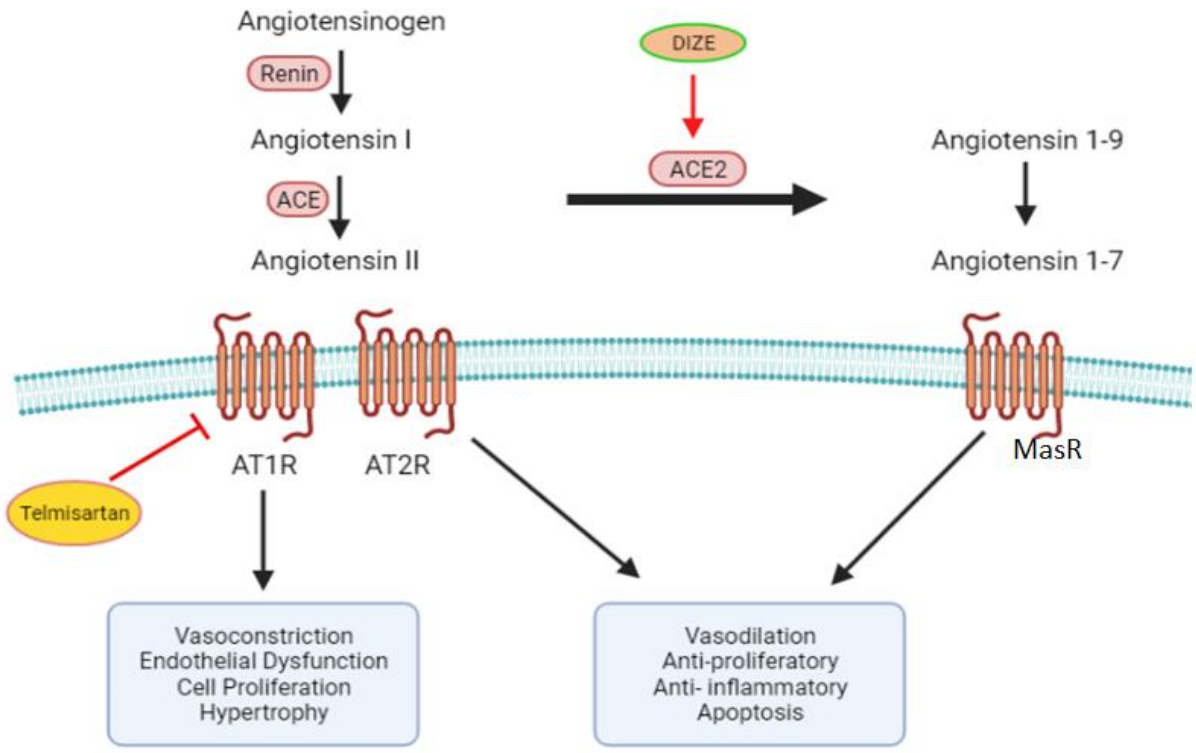


Fig. 4: Renin angiotensin system (RAS): Pressor arm and depressor arm along with their receptors and physiological and pathological functions.

2.8. Endothelial Layer and Functions

Endothelium, which is composed of a single layer of endothelial cells, is known to play a vital role in vascular homeostasis (Moncada, 2018). Vascular endothelium comprises a monolayer of simple squamous cells that form the luminal vessel wall of the whole circulatory system. Endothelial cells create the walls of capillaries and the former along with smooth muscle cells and elastic fibres make up the tunica intima of the larger blood vessels (Pi et al., 2018). Besides providing the requisite physical barrier between the blood and tissues, the endothelium serves as a sensory site, dynamically responding to mechanical and hormonal stimuli via synthesis of vasoactive peptides, which influence vasomotor activities, hemostasis, and inflammation (Cahill and Redmond, 2016). Furthermore, endothelial-derived factors interact with metabolic cells and tissues, indicating the role of endothelial cells in metabolic homeostasis (Pi et al., 2018). It also aids the functioning of the immune system by serving as a primary interface between immune cells and tissues (Khaddaj Mallat et al., 2017). Vasodilators synthesized include nitric oxide (NO), prostacyclin, C-type natriuretic peptide, hydrogen sulphide (H_2S) and various endothelium-derived hyperpolarizing factors. Vasoconstrictors include angiotensin II, endothelin-1 (ET-1), thromboxane A_2, and reactive oxygen species (ROS). Inflammatory

modulators include intercellular adhesion molecule-1 (ICAM-1), NO, vascular adhesion molecule-1 (VCAM- 1) and NF- κB. Tissue plasminogen activator, plasminogen- activator inhibitor-1, fibrinogen, von Willebrand factor tissue factor inhibitor, NO, prostacyclin and thromboxane A_2 are the factors released to regulate homeostasis (Jamwal and Sharma, 2018; Moreira et al., 2018; Yang et al., 2008). The endothelium is also involved in cell and blood vessel growth, immunological reactions and maintenance of fluid balance (Moreira et al., 2018). NO derived from the endothelium governs organ growth and hypertrophy (Jamwal and Sharma, 2018). Over the years, the endothelium is gaining recognition for the critical roles it plays in regulating physiological balance and is evolving as a potential target for various disease states.

2.9. Risk Factors Causing Endothelial Dysfunction

Holistically endothelial dysfunction is featured as an impaired vasomotor, proinflammatory and prothrombotic state, caused by any offset in the existing equilibrium that leads to detrimental functional and anatomical changes. Vascular endothelial dysfunction is indicated as a valid surrogate marker for atherosclerotic diseases and associated mortality (Demeyer and Herman, 1997). The associated clinical burdens, however, are not only limited to cardiovascular diseases such as coronary artery disease (CAD) and myocardial ischemia but extend to acute renal failure. Recent studies emphasize the systemic characteristic of endothelial dysfunction that encompasses other organ systems (Halcox et al., 2002; Widlansky et al., 2003). The pathogenesis is multifaceted and involves numerous signalling cascades. The most acknowledged include uncoupling of endothelial NO synthase (eNOS), oxidative activation of the ET-1 system, nitration and inactivation of prostacyclin synthase and direct inactivation of NO, leading to its decreased bioavailability (Vanhoutte et al., 2017). In turn these lead to a series of events e.g., increased chemokine release, overexpression of adhesion molecules, increased permeability, VSMC migration and proliferation, LDL oxidation and platelet activation. Mitochondrial ROS triggered by a state of inflammation further causes cell apoptosis via cytochrome-c release (Scioli et al., 2020). These molecular changes may be attributed to numerous diverse risk factors which range from altered metabolism and associated changes in glucose, homocysteine or lipid levels, hypertension, disrupted insulin signalling, environmental effects, aging and drug exposure (Corban et al., 2019; Daiber et al., 2017). Vascular endothelial damage was implicated in fatal and severe cases of COVID-19 due to virus induced cytokine storm production and subsequent hypercoagulation (Amraei and

Rahimi, 2020; Pons et al., 2020). ER stress often acts as the cue for the release of extracellular vesicles, ubiquitous in conditions of metabolic imbalance, which culminates in impairment of the endothelium (Osman et al., 2020). Interestingly, modifications at the epigenetic level influenced the endothelium mediated responses to hemodynamic forces (Levy et al., 2017). In the current setting, studies aim to investigate any crosstalk between dynamic signalling pathways as they could provide insights to previously unexplored potential targets.

2.10. ER Stress and Endothelial Dysfunction

ER is a dynamic membranous cell organelle that governs/oversees protein production, transport, and folding. It is integral for cellular homeostasis, as its functions also span lipid synthesis, calcium homeostasis and glucose metabolism (Cimellaro et al., 2016). Protein folding is deemed to be of significance due to the acquisition of certain conformations, which enable the proteins to act in a specified manner. External fluctuations can disfigure this intrinsic balance and set off a series of events that lead to accumulation of misfolded proteins and generation of a pre-pathological state known as ER stress (Battson et al., 2016). UPR, a safeguarding system works to diminish the ER stress and reinstate protein homeostasis by enhancing degeneration of misfolded proteins, stalling protein translation, and recruiting ER chaperones to aid in accurate folding. Accumulation of misfolded proteins acts as a cue for UPR commencement which consists of activation of three main transmembrane signal transducers which are; PKR- like ER kinase (PERK), inositol-requiring enzyme (IRE)-1α (Suganya et al., 2018), and activating transcription factor (ATF)-6 via the dissociation of these sensors from BiP (Kassan et al., 2012). PERK undergoes autophosphorylation and oligomerization, leading to phosphorylation of eIF-1α, which halts global protein translation, excluding a few proteins for example ATF-4, which governs the genes responsible for the reestablishment of ER stress. The third sensor IRE1α enhances the quality of protein synthesis by promoting the splicing of the mRNA of the transcription factor XBP1, which stimulates critical genes in the UPR system. Another shielding mechanism, autophagy is enabled by IRE1-1α, activated JNK and PERK- ATF4 pathways (Dong et al., 2017). Chronic ER stress on the other hand differs from the adaptive response triggers the UPR to function in disarray. Creation of a pro-apoptotic state occurs through the PERK- ATF4 pathway and subsequent stimulation of CHOP, which acts along with ATF4 and XBP1 (Cunard, 2017).

ER Stress has been acknowledged to be a confocal point in many pathomechanisms that lead to chronic diseases. Recently ER stress was accredited to play a role in the pathogenesis of

endothelial dysfunction, and intrinsic factors such as high protein load and dynamicity of these cells render them vulnerable to ER stress. Initial studies demonstrated that long-term UPR activation led to ED and administration of inhibitors of ER stress such as tauroursodeoxycholic acid (TUDCA) and 4-Phenylbutyric acid (4-PBA), could potentially reverse endothelial dysfunction, leading to the possibility of targeting ER stress signaling pathways in the mitigation and prevention of endothelial dysfunction and associated diseases. Studies highlight the role of specific components of the UPR machinery that contribute to endothelial dysfunction (Maamoun et al., 2019b; Scioli et al., 2020).

XBP1 was found to play both positive and negative roles in the endothelium subject to the absence or presence of ER stress response, respectively. In the absence of ER stress, the transcriptional factor is integral for endothelial cell proliferation and migration in addition to the expression of eNOS (Incalza et al., 2018; Lenna et al., 2014). However, in the presence of ER stress response and the activation of the UPR, XBP1 overexpression was revealed to be detrimental by inducing apoptosis of endothelial cells and reducing expression of VE-cadherin, which functions as a discrete adhesion molecule in the endothelial junctions stimulating the caspases signaling (Galán et al., 2014).

Endothelium dependent contractile responses in the aorta of hypertensive rats possessed an imbalance between endothelium dependent relaxing factors and constricting factors which was abolished upon treatment with ER stress inhibitors TUDCA and PBA. The ER stress inhibitors restored normal vascular tone, the molecular mechanism for which was attributed to the ability of these chemical molecules to subdue cPLA-COX pathway, thereby re-establishing balance (Lau, Yeh Siang et al., 2018). Endothelial cells of the coronary arteries were subjected to chemically-induced ER stress by tunicamycin and displayed decreased functioning and expression of eNOS, which could be attributed to the ER stress-induced phosphorylation of MAPK p38, which in turn blocked the phosphorylation of eNOS. Along with this tunicamycin-induced ROS generation depleted NO availability, further causing endothelial injury, which was negligible upon administration of TUDCA. ER stress was found to facilitate hyperglycemia-induced endothelial dysfunction, and it was evidenced by higher levels of ER stress markers including IRE-1α, PERK and XBP1 in several preclinical studies (Basha et al., 2012; Legeay et al., 2020; Sheikh-Ali et al., 2010; Suganya et al., 2018). Furthermore, the endothelium displayed overexpression of VCAM-1 and ICAM-1 along with impairment in its relaxation abilities in the case of db/db mice which were reverted with the aid of TUDCA (Battson et al., 2017). In addition to targeting the UPR to combat ER stress, focusing on

activating the AMPK an enzyme crucial for energy homeostasis has been approached for treating endothelial dysfunction. For instance, chemical activators of AMPK like metformin and aminoimidazole carboxamide riboside demonstrated their ability to diminish ER stress and associated endothelial dysfunction (Chen, C. et al., 2019).

2.11. RAS and Endothelial Dysfunction

The RAS plays a major role in governing cardiovascular homeostasis and operates discretely based on the site and level of activity. The RAS functions at the systemic, tissue, cellular and molecular level and accordingly exerts influence on inflammatory, fibrotic and thrombotic pathways in multiple organs in addition to blood pressure regulation (Lüscher, 2000). The RAS comprises two signatory axes; the classical or pressor arm and the non-classical axis or the depressor arm, which work hand in hand under normal physiology, allowing a balance between the pro-inflammatory, vasoconstrictive and proliferative actions and anti-inflammatory, vasodilatory and anti-proliferative actions, respectively (Flavahan et al., 2016). Angiotensin-II, an octapeptide that acts on the AT1 and AT2 receptors, is produced from renin aided by angiotensin-converting enzyme (ACE) that represents the classical RAS. The predominant non-classical pathway includes the ACE2/angiotensin-(1–7/Mas receptor axis, which counters the pressor actions of angiotensin II (Arendse et al., 2019). Vascular RAS promotes contraction and cell growth by the generation of angiotensin- II, mediated by ACE expressed on the endothelial cell membrane (Nair et al., 2018). Angiotensin-II stimulates NADPH oxidase, which leads to the generation of ROS. Overactivity of the pressor axis of RAS causes oxidative injury to the vessel wall and infiltration of inflammatory cells, which ultimately adversely influences endothelial cell regeneration and vascular remodelling owing to dysfunctional endothelial progenitor cells (EPCs). The activity of the latter diminishes upon angiotensin-II mediated AT1R activation and subsequent signalling (Becher et al., 2010). An imbalance between angiotensin-II, a prominent vasoconstrictor molecule and other vasodilatory molecules such as endothelium derived NO transpires during the development of endothelial dysfunction. This disparity is a crucial element during the pathogenesis of endothelial dysfunction, which occurs due to the unregulated actions of angiotensin-II such as inhibition of NO synthetase and promotion of endothelin converting enzyme, decreasing NO availability and increasing ET-1, respectively (Batenburg et al., 2012).

Experimental studies have implicated the crosstalk between certain components of the RAS pathway with other mediators of endothelial dysfunction. ACE gene upregulation along with overexpression of ACE and subsequent rise in angiotensin-II levels were found to be

quintessential for endothelial dysfunction contributed by increased levels of 20-hydroxyeicosatetraenoic acid (20-HETE), an angiogenic eicosanoid present prominently in the vasculature of the brain and kidney (Cheng et al., 2012). Aldosterone mediated endothelial dysfunction was found to be dependent on functional AT1aR (Briet et al., 2016). Furthermore exaggerated RAS activation and insulin resistance cross intervene to result in endothelial dysfunction, implying complex molecular mechanisms (Liu, 2007). Local intravascular RAS instigates diminishing endothelium dependent vasodilation associated with aging whereas long term treatment with AT1 R blocker cerivastatin and or ACE inhibitor CS-866 demonstrated the reversibility potential. The mechanism of action was attributed to the effects the drug molecules had on decreasing the expression of COX-2 in the vasculature and subsequent decline in vasoconstrictor eicosanoids and superoxide anions (Mukai et al., 2002).

Inhibitors of the pressor axis of RAS have been therapeutically explored for their potential to overcome disorders associated with endothelial dysfunction. For instance, RAAS blockade was found to impede atherosclerosis in animals with pre-existing diabetes (Cooper, 2004). RAS overactivation and subsequent oxidative stress have been demonstrated to contribute towards endothelial dysfunction associated with estrogen deficiency in ovariectomized rats. This impairment, however, was seen to improve upon the administration of an AT1 R antagonist, Losartan or an ACE inhibitor enalapril (Yung et al., 2011). Captopril, an ACE inhibitor, prevented endothelial cell damage and enhanced their revival in coronary arteries in vitro by interrupting the Akt/mTOR pathway which is responsible for cellular apoptosis induced by ROS (Shi et al., 2019). AT1 receptor antagonist TCV-116, ACE inhibitor enalapril or the combination of the two were effectively able to re-establish the dysfunctional dilations which were endothelium-dependent when administered to spontaneously hypertensive rats (SHRs), signifying the potential of RAS blockade in alleviating endothelial dysfunction in hypertensive patients (Goto et al., 2000). Angiotensin II receptor–neprilysin inhibitor sacubitril/valsartan (LCZ696) and valsartan were comparable in their ability to ameliorate endothelium-dependent relaxation impairments in SHRs (Seki et al., 2017). AT1R antagonist losartan and ACE inhibitor were established to counter impaired endothelium-dependent relaxation in human subjects who were subjected to atypical FFA levels depicting the role of RAS in abnormal lipid-induced endothelial dysfunction (Watanabe et al., 2005).

The protective arm of the RAS, ACE2/angiotensin (1-7)/Mas receptor axis, is recently being sought to identify potential drug targets to combat chronic diseases linked to endothelial dysfunction (Radenkovic et al., 2016). Studies have underlined the significance of angiotensin (1-7) in both exogenous and endogenous forms and ACE2 expression and activation in

restoring the function of EPCs and consequent reparation of vascular endothelial damage that occurred in diabetic individuals (Jarajapu et al., 2013; Willemsen et al., 2007). Resorcinolnaphthalein diminished endothelial dysfunction and lessened the severity of associated pulmonary arterial hypertension by activating ACE2 (Li, G. et al., 2017). The 1-(Fraga-Silva et al.)amino]-4-(hidroximetil)-7-(Fraga-Silva et al.)oxi]-9H-xantona-9 (XNT), another ACE2 activator, improved the endothelial function as evidenced by enhanced vasorelaxations of the aortas isolated from in SHR and diabetic rats via activating the Mas receptor and reducing oxidative stress (Fraga-Silva et al., 2013). Xanthenone by virtue of its ACE-2 activation, was capable of reverting the gestational proteinuria and hypertension induced by leptin through endothelial activation (Ibrahim et al., 2014). Together, inhibiting the pressor axis or stimulating the depressor axis of the RAS has exhibited the potential to mitigate endothelial dysfunction and thus prevent or dampen the progression of varying linked disorders.

2.12. ER Stress and RAS Crosstalk in Endothelial Dysfunction

ER Stress and different components of RAS are well known to impair endothelial function, as discussed above. Moreover, there exists vital crosstalk between ER Stress and RAS components which is highlighted in a few of the studies. Murugan et al. reported that angiotensin-II led to the elevation in ER stress, which caused an impairment in vascular function. ER Stress inhibitors such as 4-PBA and TUDCA effectively prevented the angiotensin-II induced endothelial dysfunction, corroborating that angiotensin-II caused endothelial impairment by increasing ER stress. Besides, they also found that angiotensin (1-7), protective component of RAS, also protected the endothelium from damage via reducing ER stress (Murugan et al., 2016). In another study, arsenic exposure led to the elevation of different RAS components, including angiotensinogen, angiotensin-converting enzyme (ACE, AT1R and angiotensin II in human umbilical vein endothelial cells (HUVEC). Further arsenite exposure caused an augmentation in ER stress via IRE1a/XBP1s pathway of the UPR, which resulted in the activation of RAS signaling pathway leading to endothelial damage. Silencing of the IRE1a/XBP1s reversed the damage indicating the vital role played by ER stress in augmentation of angiotensin-II signaling and the resulting endothelial dysfunction (Xu et al., 2017a). A similar cross-talk between IRE1α/XBP1s branch of UPR with angiotensin-II signalling pathway was found in a study evaluating the effect of particulate matter (PM) 2.5 exposure on vascular endothelium (Xu et al., 2017b). In this study, they reported that IRE1α/XBP1s branch of UPR was in fact responsible for the upregulation of the angiotensin-

II signalling pathway in the endothelial cells exposed to PM 2.5, leading to their damage (Xu et al., 2017b). Two preclinical studies exploring the activity of metformin in the prevention of hypertension found that angiotensin II infusion in mice, besides exposure of HUVEC cells to the same, led to the development of vascular ER stress thus leading to hypertension (Chen, C. et al., 2019; Duan and Song, 2017). Both these studies stressed the fact that elevated angiotensin -II leads to aberrant ER stress development, which may further be responsible for the damage to the endothelium. ER stress was also found to mediate angiotensin induced endothelial dysfunction via other mediators. Mak et al. reported that ER stress was involved in the upregulation of soluble epoxide hydrolase on angiotensin-II exposure in the coronary endothelium, causing injury to the same (Mak et al., 2017). Thus, most of these studies depict that angiotensin-II may cause endothelial damage by other patho-mechanisms, including ER stress (Fig. 5) (Table 1).

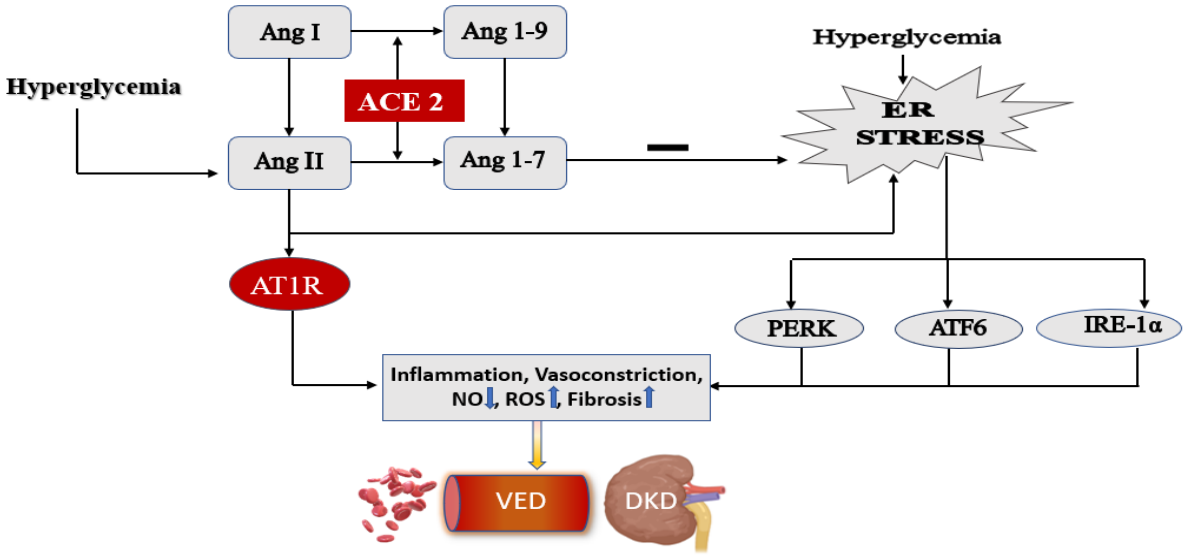


Fig. 5: Renin angiotensin system (RAS) and ER stress crosstalk in endothelial dysfunction. RAS has two arms one is the vasoprotective arm which comprises of ang (1-7) which is made up from angiotensin-II and angiotensin (1-9) by the enzyme angiotensin converting enzyme 2 (ACE2). Ang (1-7) can reduce ER stress. The other arm of the RAS consists of angiotensin-II which acts via angiotensin type 1 receptor and is deleterious to the vascular endothelium. ER stress could be caused by external factors as well as by modulation of RAS, primarily increased ang-II. **Ang:** Angiotensin; **AT1R:** Angiotensin type 1 receptor; **NO:** Nitric oxide; **ROS:** Reactive oxygen species; **PERK:** protein kinase R (PKR)-like endoplasmic reticulum kinase; **ATF6:** Activating transcription factor 6; **IRE1α:** Inositol-requiring enzyme 1 α.

Table 1: ER Stress and RAS Crosstalk in Endothelial Dysfunction

S. No	Chemical/ peptides	ER stress inhibitor used	Animals/ Cells	Targeted pathway	Outcome of study	Ref.
1	Ang 1-7	TUDCA and 4-PBA	C57BL/6J mice/ HUVECs	MasR/eIF2-α	Ang 1-7 attenuates Ang-II induced ER stress and reduces endothelial dysfunction via Mas receptor	(Murugan et al., 2016)
2.	Arsenic induced ER stress	-	HUVECs	IRE1a/XBP1s	Activated IRE1a/XBP1s pathway is responsible for elevation of RAS components	(Xu et al., 2017a)
3.	Particulate matter PM 2.5 induced ER stress	-	Male SD rats/ HUVECs	IRE1α/XBP1s	Exposure of PM 2.5 upregulates IRE1α/XBP1s that elevates Ang-II signaling	(Xu et al., 2017b)
4.	Metformin	TUDCA	WT C57BL/6J mice/ hVSMCs	AMPK	Metformin treatment reduces Ang II induced ER stress	(Chen, C. et al., 2019; Duan and Song, 2017)
5.	Tetramethylpyrazine	TUDCA and 4-PBA	Pigs heart/ PCECs	Ang-II induced soluble epoxide hydroxylase	ER stress regulates level of Ang-II induced soluble epoxide hydroxylase	(Mak et al., 2017)

2.13. Epigenetics

Epigenetics can be simply put as the instructions and information in addition to that encoded in the DNA or the genome which is consistent throughout the cells. Unlike the genome of an organism, epigenome is not a consistent entity and may be modulated by different intrinsic, chemical and environmental cues (Kanherkar et al., 2014; Riancho et al., 2016). Not only this, these changes could be inherited across generations (Lind and Spagopoulou, 2018). The changes in the epigenetics of eukaryotes can be majorly attributed to Histone modifications, DNA methylation, and RNA associated silencing (Allis and Jenuwein, 2016; Handy et al., 2011; Kanherkar et al., 2014; Kelly et al., 2010).

DNA methylation is an epigenetic modification associated with silencing of the genes occurring on CpG dinucleotide sites. It is carried out by a set of enzymes called DNA methyltransferases whose primary role is to transfer a methyl group from S-adenyl methionine to cytosine at position C5 forming 5-methylcytosine (5-mC) (Moore et al., 2013; Villota-Salazar et al., 2016). Coherency in the maintenance of the methylation pattern is vital for embryonic development, cell proliferation and other physiological processes validated by different pathologies occurring due to an imbalance in the DNA methylation machinery (Jones, 2012).

Histones are basic proteins around which the DNA wraps itself generating chromatin. Nucleosome can be considered as the first subunit of the chromatin with two copies each of histones H2A, H2B, H3, and H4, congregated in an octameric fashion with ~200 bp of DNA coiled around it. Thus, histone plays an important role in the packaging of the long DNA compactly inside the structural unit nucleosome and in gene expression regulation (Dahlby et al., 2020; Fu et al., 2019). Being the core component of the nucleosomes, histones are subjected to a myriad of post-translational modifications (PTMs) also called as histone marks. They are subjected to in excess of ~130 posttranslational modifications majorly including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation etc (Chen et al., 2017). Impact of histone acetylation, methylation, and phosphorylation on gene expression has been thoroughly investigated but recently several other modifications viz. propionylation, butyrylation, crotonylation, malonylation, and succinylation of the lysine residues has come to the fore and their effect is distinct from those of lysine acetylations (Huang et al., 2014). All the above post-translational modifications are believed to modulate DNA transcription and gene expression.

In recent past already few HDAC inhibitors (vorinostat, panobinostat) has been approved by US-FDA for cancer treatment and studies are in process across the globe to identify and explore

the use of HDAC inhibitors in different diseases including cancer, neurological disorders, cardiovascular disorders etc. (Fang et al., 2013; Yoon and Eom, 2016). Further in detail description of abovementioned and other epigenetic processes are out of the scope of this review and has been reviewed thoroughly elsewhere.

2.14. Epigenetics and ER Stress

We have already discussed regarding the importance of ER stress and UPR in different diabetic complications. This brings us to the regulation of ER stress in different diseases conditions. One of the important regulators of the ER stress is the epigenetic machinery. ER stress response and, thus, disease risks may be regulated by different epigenetic modifications in addition to environmental factors. A number of DNA and histone methylation, acetylation patterns were observed in or near ER stress gene promoters and downstream signaling molecules. In addition, long non-coding RNAs and microRNA (miRNA) expression could be a significant determinant of ER activity under stress conditions. Transcriptional factors controlling the UPR include ATF4, ATF6, XBP1, CHOP, and JUN. It is known that members of this family can form homotypic and heterotypic dimers; therefore, different genes may be regulated by each complex (Barroso and Chevet, 2016). A gene expression pattern can be influenced by epigenetic modifications. There is a possible link between epigenetic changes and the pathogenesis of diabetes complications in this gene-environment interaction (Chen, J. et al., 2014). Researchers are continuously exploring the link between ER stress and the epigenetic changes and have found that different epigenetic components are involved in the regulation of ER stress.

Sun et al., in their report verified the role of epigenetic components in the regulation of ER stress during DKD. They explored the role of histone acetylation modification in the regulation of the genes which are privy to the modulation of ER stress. In the study, they used diabetic rats who were administered valproate (a non-selective HDAC inhibitor). In the DKD animals ER stress and apoptosis was increased as evidenced by higher expression of CHOP, caspase-3, caspase-12. The animals treated with Valproate demonstrated reduced ER stress and apoptosis. They also showed that valproate regulates the acetylation of histone H4 in the promoter of GRP78 and suppresses the histone H4 acetylation on the promoter of CHOP using Chip assay. The study reveals the role of HDAC, in the regulation of ER stress during DKD besides demonstrating that epigenetic modifier in the form of valproate could turn to be a useful tool against DKD (Sun et al., 2016). The role of valproate in reducing the hyperglycaemia induced ER stress was further validated by Sun et al. They used the rat tubular epithelial cells

(NRK-52E) and exposed the same to hyperglycaemic environments which led to an increase in ER stress. Further they showed that valproate exposure led to increased H4 acetylation on the promoter of GRP78 in NRK-52E cells (Sun et al., 2020). In a similar set of studies, selective inhibition of HDAC3 using BRD3308 and CI-994 inhibited the ER stress induced β cell apoptosis implicating towards the regulation of ER stress by another HDAC (Wagner et al., 2016). One of the most commonly explored HDAC, SIRT1 was found to regulate the ER stress in diabetic ischemic rats. The diabetic ischemic rats showed significantly reduced SIRT1 expression which was followed by increased ER stress and renal dysfunction. On the administration of a SIRT1 agonist the rats showed improvement along with reduction in ER stress indicating towards the regulatory role of SIRT1 on ER stress (Zhang et al., 2020). Another report found that selective inhibition of HDAC6 using 23BB alleviated rhabdomyolysis related acute kidney injury by regulating the ER stress and apoptosis (Feng et al., 2018).

Apart from histone acetylation, histone methylation and the enzymes mediating the methylation, histone methyltransferases were also reported to regulate ER stress. One of the histone methyltransferases, Enhancer of zeste homolog 2 (EZH2) was found to be involved in the glucolipotoxicity induced ER stress in pancreatic β- cells. Small molecule inhibition as well as knockdown of the EZH2 led to inhibition of ER stress and resulting β- cell apoptosis. Another histone methyltransferase SET7/9 was found to induce methylation in diabetic kidneys due to ER stress (Dahlby et al., 2020). Chen et al., reported that silencing of the XBP1s or SET7/9 gene using specific siRNAs markedly changed the ER stress induced elevation in SET7/9, H3K4Me1 in DKD (Chen, J. et al., 2014). This shows that regulation of ER stress via different histone methyltransferases.

2.15. Epigenetics and Diabetic Kidney Disease

DKD has a complex multifactorial etiology which is no way limited only up to physiological processes but also includes genetic and epigenetic or environmental factors as evidenced by the fact that only ~30% of the diabetic patients suffer from renal pathologies in due course of time. Researchers around the world are working on exploring the relationship between DKD and genetic makeup using techniques like fine linkage mapping, genome-wide association studies (GWAS) etc (Liu et al., 2015).

Wang and Sun et al performed a network analysis of available tissue-specific gene expression and methylation data and found that varying methylated states of the core regulating genes may

be a vital player in the development of DKD (Wang et al., 2018). Diabetes and hyperglycemia are found to be associated with aberrant DNA methylation (Marumo et al., 2015). In a genome-wide analysis carried out by Sapienza et al on the salivary samples of diabetic patients without nephropathy and diabetic patients with ESRD, differentially methylation was reported (Sapienza et al., 2014). Wing et al also after genome-wide methylation pattern analysis in CRIC (Chronic renal insufficiency) study found a positive association between aberrant methylation patterns with a rapid decline in renal functions (Wing et al., 2014). On similar lines EWAS (Epigenome-wide association studies) of the renal function involving 4859 subjects revealed the association of differential methylation and progression of chronic kidney disease and even renal fibrosis (Chu et al., 2017). Taking a cue from previous reports Bomsztyk et al very recently published a clinical report regarding DNA methylation status and DN in Pima Indian ethnic group. During the genomewide blood leukocyte survey, they found that DNA was differentially methylated at genes associated with DN (Bomsztyk et al., 2018). The study was similar to that of Qiu and Hanson et al in which they explored the impact of cytosine methylation on the progression of DN in Pima Indians over a period of six years. They found that cytosine methylation was positively correlated to parameters of DN progression viz estimated glomerular filtration rate (eGFR) decline and albumin: creatinine ratio (Qiu et al., 2018). Thus, it can be concluded that the DNA methylation status may prove to be a handy biomarker for prognosis of DN.

Histone post-translational modifications also play a major role in the expression of genes associated with DKD progression. TGF-β1 is known to have a role in the expression of ECM genes which is one of the factors for nephropathy development. Natarajan et al showed TGF-β1 treatment results into the enhancement of ECM associated genes viz connective tissue growth factor (CTGF), Collagen-1 and plasminogen activator inhibitor-1. The reason for this being enhancement of H3K4me1, H3K4me2, H3K4me3 (active genes) and reduction of H3K9me2 and H3K9me3 (repressive genes) in the promoter region of the aforementioned genes (Sun, G. et al., 2010). Dyslipidemia is also one of the factors leading to DKD. Further investigation by Natarajan et al revealed that elevated 12/15-Lipoxygenase modulates the histone modifications related to pro-fibrotic genes in mesangial cells (Yuan et al., 2016). Renal inflammation is also a key feature of DN development which in turn is reported to be regulated by histone acetylation. Chen et al found that rat mesangial cells exposed to hyperglycemia showed enhanced H3K9ac and H3K18ac and also HDAC1 reduction by over 55% as compared to control. Apelin-13 reduced the hyperglycemia-induced inflammation by abating

hyperacetylation of histones and restoring HDAC1 activity (Chen, H. et al., 2014). In another study by Miao and Cai et al DN related histone hyperacetylation (H3K9 and H3K14), increased histone acetyl transferase activity was reduced by C66 treatment preventing progression of nephropathy (Wang, Y. et al., 2015). Apart from acetylation and methylation of histones another histone modification, ubiquitination has come to the fore in the progression of DN. Histone ubiquitination may enhance the progression of DKD or retard its progression. We recently reported renoprotective activity of Aspirin via H2AK119 ubiquitination in diabetic animals (Goru and Gaikwad, 2018). Earlier too we reported the role of H2AK119 ubiquitination in AT-II receptor-mediated macrophage infiltration and fibrosis (Pandey et al., 2016). In another study by our lab esculetin was found to abate DN by reducing H2AK119 ubiquitination and H3 acetylation (Kadakol, Almesh et al., 2017). Post translational histone modifications and DNA methylation related to DN has been listed in Table 2. Research regarding the role of epigenetics in human diseases has grown leaps and bounds as evidenced by the fact that number of published articles involving Epigenetics rose from 2500 in 2006 to 17,000 in 2013 almost an increase of 6.8 folds (Deans and Maggert, 2015). It is fair enough to investigate the role of epigenetics in DKD since already existing pathophysiological pathways and therapies targeting them have failed to produce a positive long-lasting effect.

2.15.1. SET7/9 and its Role in DKD

Among the several posttranslational histone modifications (PTHM), histone methylation is one of the most commonly reported PTHM in kidney diseases. A number of histone methyltransferases such as SET7/9, EZH2, G9a, Suv39h1 etc. are reported to bring the methylation of histones in different kidney diseases such as DKD, CKD, AKI etc. However, SET7/9 is one of the histone methyltransferases which is slowly gaining prominence in kidney diseases. Several reports in the past decade have revealed the role of SET7/9 in the progression of varying kidney disorders projecting it as a valuable therapeutic target to prevent such diseases from progression. SET7/9 is one of the few enzymes which have been linked to the promotion of extracellular matrix proteins *in-vitro* (Sun, Guangdong et al., 2010). Sun et al., in their study elucidated that kidney mesangial cells incubated with TGF β1 showed elevation in active chromatin marks (H3K4Me1, H3K4Me2 and H3K4Me3) along with reduction in the levels of repressive marks (H3K9Me2 and H3K9Me3). More importantly, TGF β1 also enhanced the expression of SET7/9. Interestingly, inhibition of SET7/9 using siSET7/9 led to reduction in the ECM gene expression caused due to TGF β1 (Sun, G. et al., 2010).

Sasaki et al., explored the role of SET7/9 in renal fibrosis. They used UUO model of renal fibrosis and incubated NRK-52E cells with TGF β1 to study the expression of SET7/9 and the downstream signaling pathways. They observed increased expression of SET7/9 in kidney of UUO mice, NRK-52E cells incubated with TGF β1. Moreover, they also reported that SET7/9 is also one of determinants of the fibrosis in human kidney samples. Knockdown of SET7/9 using sinefungin or siRNA reversed the fibrogenesis associated with TGF β1 *in-vivo* and *in-vitro* (Sasaki et al., 2016). In another such study, the role of SET7/9 emerged in the regulation of DKD via modulating the p21 gene expression. Reports suggest that p21 gene is vital to the pathogenesis of DKD by regulating the glomerular and mesangial expansion. Li et al. explored the role of p21 in glomeruli of diabetic rats and mesangial cells incubated with high glucose. In glomeruli of the diabetic rats as well as the mesangial cells treated with high glucose p21 expression was significantly upregulated along with reduced H3K9Me2 and increased H3K4Me1/3 and SET7/9 at the promoter of p21 indicating its role in the progression of DKD (Li et al., 2016). Similar findings were reported by Chen et al., where they showed that mice and NRK-52E cells with renal ischemia/reperfusion (I/R) injury have increased expression of SET7/9 and the consequent histone methylations (Chen, H. et al., 2014). A report revealed that in CKD, downregulation of SET7/9 and autophagy activation may be the key mechanisms for vascular calcification induced by Indoxyl sulfate (Chen, J. et al., 2019).

Previously, we have also explored the role of SET7/9 in DKD as well as AKI. Goru et al., showed that H2AK119 mono-ubiquitination (H2AK119Ub) and H2BK120 mono-ubiquitination were involved in the development of renal fibrosis in T1DM animals via modulation of SET7/9 (Goru et al., 2016). Sharma et al., explored the role of SET7/9 in renal ischemia/reperfusion (I/R) injury and found that it is highly upregulated. They utilized a novel SET7/9 inhibitor Cyproheptadine which led to amelioration of AKI via SET7/9 inhibiton (Sharma et al., 2020).

Overall, these reports suggest that targeting SET7/9 could prove to be very fruitful in development of a novel therapeutic strategy against kidney diseases.

Table 2: PTHMs related to development and progression of diabetic nephropathy

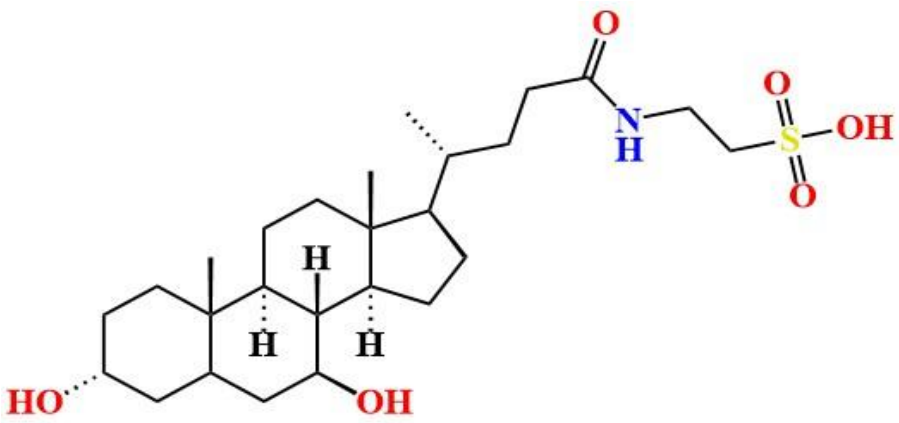
Enzyme	Study cells/Species	Target genes/proteins	Effect	References
HDAC1	RMCs; Akita mice	*MCP-1*, *ICAM-1* and *VCAM-1*	Proteinuria, glomerular hypertrophy, mesangial expansion and inflammation.	Chen et al., 2014
HDAC2	Type1 and Type 2 diabetic rats, NRK52-E cells	Fibronectin, Collagen I, α-SMA expression	ECM accumulation, EMT	Noh et al., 2009
HDAC4	Diabetic db/db mice, STZ-induced rats, diabetic patients	STAT1, Beclin 1	Proinflammatory mediator generation, Podocyte apoptosis, Inhibition of Autophagy	Wang et al., 2009
HDAC5	RMCs and mice mesangial cells	PAI-1, p21	Glomerulosclerosis and hypertrophy	Yuan et al, 2012
HAT (p300/CBP)	C57BL/6J male mice	CTGF, PAI-1, and FN-1	Renal hypertrophy and fibrosis	Wang et al, 2015
HMT (SET7/9)	Rat Mesangial Cells	Col1a1, CTGF, and PAI-1	Increased fibrotic genes expression	Sun G., et al, 2010
HMT (SET7)	Rat mesangial cells; STZ mice	*Serpine1*, *CTGF*, and *Col1a1*	Increased fibrotic genes expression; Proteinuria, ECM deposition, fibrosis and glomerulosclerosis	Yuan et al., 2016

2.16. Nutraceuticals as a Combination Therapy in Diabetic Complications: Emphasis on TUDCA as a supplement during DKD

Over the past two-to-three decades, conventional and complementary medicines have been integrated with healthcare. Particularly the chronic diseases where dietary and lifestyle interventions could modulate disease outcome has been the focus of research in the recent times. Diabetic patients have to be on anti-diabetic medications for majority of their lives and still the condition goes on progressing requiring escalation of doses or requiring a combination of anti-diabetic drugs, also termed as polypharmacy. The routine treatments are also linked to several side-effects ranging from extreme fluctuation in patient weight, hypoglycaemic episodes to name a few. Although there are several medications available for glycaemic control, the number of patients using additional dietary supplements either *per se* or in addition

to the existing therapies is high. As per a survey report, 57% of the participating diabetic people agreed to have consumed complementary alternative medicine (Yeung et al., 2018). Traditional Chinese medicines and herbs have been researched for decades in preventing the progression of diabetes and associated complications. Curcumin, Berberine, Resveratrol, Quercetin etc. have been mentioned frequently in literature regarding their anti-diabetic activity. Moreover, reports also suggest that combining the traditional complementary therapy with one of the conventional medicines produced better outcomes.

TUDCA is one such naturally derived dietary supplement which is now explored for its therapeutic potential in different diseases including T1DM, T2DM, Alzheimer's and other neurodegenerative disorders (Fig. 6). Traditionally, bear bile acid powder was the primary source of TUDCA. This powder is made from bile collected from bears living in the Ursidae family, like the black bear (Selenarctos thibetanus) and brown bear (Ursus arctos) which are both endangered. Presently, bear bile powder for medicinal use is extracted from farmed bears using the "Free-dripping Fistula Technique" by implanting a duct or making an artificial fistula in their liver. This method is cruel and would result in their death due to chronic infections or liver cancer.



Tauroursodeoxycholic acid

Molecular formula: $C_{26}H_{45}NO_6S$

Molecular weight: 499.7

Fig. 6: TUDCA structure and molecular formula

As discussed earlier, ER stress is one of the primary mechanism responsible for the progression of diabetes and associated complications. TUDCA is known for its chaperoning activity via which it reduces ER stress. There are several reports which propose the ER stress inhibitory activity of the chemical chaperone, TUDCA including in DKD. Zhang et al., in their study explored the role of TUDCA in preventing the renal tubular damage associated with T2DM. Earlier, glomerular injury was considered hallmark to DKD however now the role of tubulointerstitial fibrosis is also recognized in the progression of DKD. Zhang et al., reported that in streptozotocin treated male db/db mice ER stress played vital role in tubular cell apoptosis and diabetic nephropathy progression. To mitigate ER stress and prevent the progression of diabetic nephropathy they administered TUDCA in the dose of 250 mg/kg twice a day for 8 weeks. The TUDCA treated group showed reduced damage to the kidney. In recent times, several other studies have projected the protective role of TUDCA in DKD. TUDCA is not only known to be effective in biliary and kidney diseases but are explored for its protective role in several neurodegenerative and metabolic disorders. Since TUDCA is a bile derivative, and in therapeutic use, bile acids can be administered orally, subcutaneously, or intravenously. Additionally, they typically present relatively low toxicity to the organism as well as the ability to cross the blood-brain barrier (Kusaczuk, 2019). Due to these favourable characteristics, TUDCA has been studied as a potential therapeutic agent in a wide range of diseases. Studies have revealed a completely novel aspect of TUDCA functioning in which epigenetic mechanisms are modified inside a cell. In a study, it was found that treatment with TUDCA resulted in a significant increase in global level of acetylated histone 3 (H3K9ac) (Zhang Y et al, 2018). Moreover, in another set of studies involving experimental model of ethanol exposure prenatally TUDCA restored the dysregulated expressions of HDAC1, 3, 4, 5, 7, and 9 (Yao X, et al., 2014). Also, another report said that by inhibiting class I and class II HDAC enzymes by TUDCA, insulin sensitivity and glucose tolerance were improved (Yao X, et al., 2013). It is well known that modulating the epigenetic machinery of cell leads to several changes and may help in regulating the cell cycle, cell apoptosis, differentiation and senescence. It could be a possible hypothesis that epigenetic regulation by TUDCA provides it the pleiotropic activity.

Overall, the ER chaperoning activity combined with several other potential protective molecular mechanisms TUDCA emerged as a valuable therapeutic entity which should be further explored *per se* as well as in combination with existing therapies.

Chapter 3

Background and Objectives

3.1. Background

ER stress is one of the main culprits in the progression of both T1DM and T2DM and the associated complications including endothelial dysfunction. However, the role of ERS in the development and progression of DKD has gained the limelight in the past few years and the exact pathway is still not known. Any impairment in protein synthesis, folding, and degradation by the ER causes ER stress which in turn leads to activation of unfolded protein response (UPR) to maintain the homeostatic environment. Targeted alleviation of ERS is seen as a novel therapeutic target to arrest the progression of DKD.

Epigenetic modifications have been vital in altering gene expression patterns. This gene-environment interaction involving epigenetic changes may be particularly relevant to the pathogenesis of diabetic nephropathy. Epigenetic modifications are reported to regulate UPR, including activation/synthesis of transcription factors, recruitment of transcription complex, and changes in chromatin histone marks. Therefore, modulating epigenetic machinery may help in arresting the progression of DKD via regulating the renal ER stress.

Tauroursodeoxycholic acid (TUDCA), bile salt, a nutraceutical and a chemical chaperone is reported to reduce ERS. Telmisartan, an AT1R antagonist, is well known for its renoprotective actions in DKD. However, the exact mechanism is yet to be found. Telmisartan is reported to alleviate oxidative stress, ERS, and induce autophagy via the PPAR-γ pathway apart from inhibiting Angiotensin II (Ang II) activity which itself is an initiator of ERS. Telmisartan is a potential candidate for concomitant administration with TUDCA in ameliorating DKD owing to its pleiotropic effects. Also, the promising molecule Diminazene aceturate (DIZE), an ACE-2 activator is reported to act through the protective axis of the RAS i.e., ACE2-Ang (1-7)-Mas receptor axis. There has been also significant evidence regarding the utility of ACE2-Ang (1-7) in the prevention of endothelial dysfunction, but the work is still in progress and needs further evaluation. The role of RAS is well known in the development and progression of DKD. The existing therapies which mainly include AT1R antagonism are not enough to arrest the progression of DKD which is a condition characterized by multiple etiologies and thus requires to add on therapies to target different pathways involved and hence achieve the desired outcome. ARBs have been effective in countering endothelial dysfunction but the role of the protective arm of RAS needs to be evaluated in endothelial dysfunction. ERS is also reported to interfere with ACE2 and Ang (1-7) via mechanisms that are not clearly known. ERS inhibition may have a positive impact on RAS. We intend to study the role of epigenetic regulation of renal ER stress and modulation of the same in preventing the progression of DKD.

We also intend to study the potential role of ERS inhibitor (TUDCA) as an add-on therapy for renin-angiotensin system modulation [ACE2-Ang (1-7) axis and AT1R blockade] in diabetic vascular endothelial dysfunction and DKD respectively.

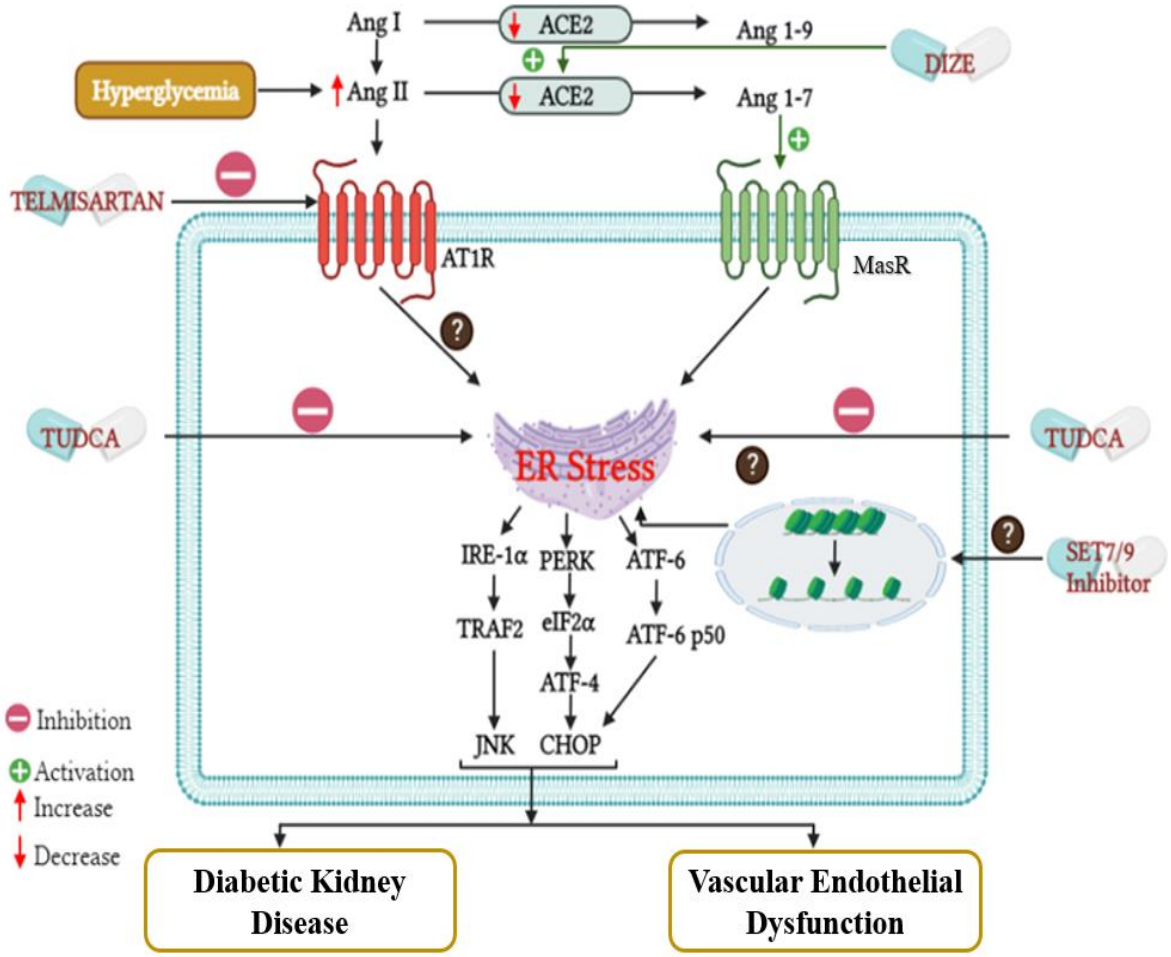


Fig. 7: Gaps in existing research concerning ER stress in development and progression of DKD and vascular endothelial dysfunction under T1DM.

3.2. Objectives

- To study the effect of ER stress inhibitor (TUDCA) and ACE2 activator (DIZE) combination therapy in the prevention of vascular endothelial dysfunction under type 1 diabetic condition.
- To study the epigenetic regulation of ER stress in kidney disease under type 1 diabetic condition.
- To evaluate the efficacy of ER stress inhibition by TUDCA as an add-on therapy to AT1R antagonist (Telmisartan) against the development and progression of kidney disease under type 1 diabetic condition.

Chapter 4

Materials and Methods

4. 1. Materials

All the instruments and materials used throughout the study were enlisted in the below tables ***(Table 3 and Table 4)***.

Table 3 List of instruments used in the study.

#	Name of the instrument	Make	Country
1.	Biosafety Cabinet (BSL2)	Thermo-Scientific	Australia
2.	CO_2 incubator	Thermo-Scientific	USA
3.	Inverted Microscope	Zeiss-Vert	Germany
4.	Rotary Operated Manual Microtome	Leica Biosystems	Germany
5.	Mini-PROTEAN® Tetra Cell Vertical electrophoresis unit	Bio-Rad	USA
6.	-86°C Ultra-Low Temperature Upright Deep Freezers	Thermo Fisher	USA
7.	C1000 Touch™ Thermal Cycler	Bio-Rad	USA
8.	Tras-Blot® SD- Semi-Dry transfer apparatus	Bio-Rad	USA
9.	Chemic Doc XRS+	Bio-Rad	USA
10.	LightCycler® 96-RT-PCR System	Roche	Germany
11.	Flow Cytometer	Beckman Coulter	USA
12.	Confocal Microscope	Zeiss	Germany
13.	2- Chamber Organ Bath and Force Transducer	Ugo-Basile	Italy

Table 4 Drugs and Biochemical Kits

#	Name of the product	Suppliers
1.	Streptozotocin	Sigma-Aldrich India (Delhi, India)
2.	Cyproheptadine hydrochloride	Tocris Biosciences, (Bristol, UK)
3.	Diminazene Aceturate	Sigma-Aldrich India (Delhi, India)
4.	Tauroursodeoxycholic acid, Sodium	Merck (Darmstadt, Germany)
5.	Biochemical estimation kits for glucose, urea, creatinine, albumin etc	Accurex Biomedical Pvt. Ltd. (Mumbai, Maharashtra, India).

4.2. Animal Studies

All the animal studies were carried out as per the guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and Institutional Animal Ethics Committee, Birla Institute of Technology and Science, Pilani (BITS Pilani) [IAEC/RES/25/14/Rev-1/27/15]. The animal experiments were also performed in accordance with the ARRIVE guidelines.

4.3. Type 1 Diabetes Induction in Rats

T1DM was induced in male Wistar rats (~250 gm) by administration of a single dose of streptozotocin (STZ) [55 mg/kg, *i.p.*] dissolved in sodium citrate buffer (0.01 M, pH 4.4) (Goru, Santosh Kumar et al., 2017). Normal control (NC) rats were administered with an equal volume of sodium citrate buffer. Blood glucose was evaluated after 48 hrs of streptozotocin induction and the animals with blood glucose in excess of 13.9 mmol/L were considered diabetic and included in the study.

Study Plans and Treatment Regimens

For study 1:

In the first objective, the potential of the combination of an ER stress inhibitor (TUDCA) and ACE2 activator (Diminazene aceturate) in preventing diabetes-induced vascular endothelial dysfunction was studied. To perform this objective 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 $mg \cdot kg^{-1} \cdot day^{-1}$, *p.o.*) (Goru, Santosh Kumar et al., 2017) (iv) DC animals were administered with TUDCA (200 $mg \cdot kg^{-1} \cdot day^{-1}$, *i. p.*) (Choi et al., 2016) (v) DC animals were treated with a combination of diminazene aceturate (5 $mg \cdot kg^{-1} \cdot day^{-1}$, *p.o.*) and TUDCA (200 $mg \cdot kg^{-1} \cdot day^{-1}$, *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 (Fig. 8).

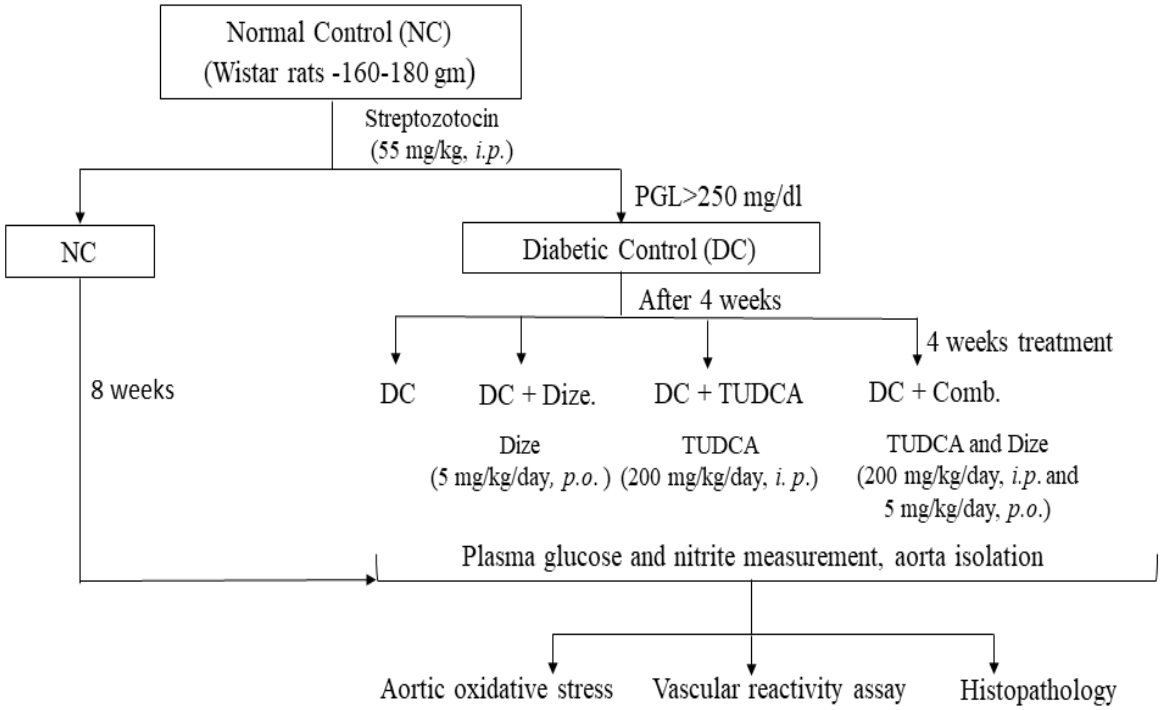


Fig. 8: Schematic representation for the methodology of study 1.

For study 2:

The second objective was based on the exploration of the regulation of ER stress in the hyperglycemic condition in NRK52E cells which mimics the *in-vivo* DKD pathophysiology. The objective primarily involved *in-vitro* studies which was conducted as follows. Rat proximal renal tubular epithelial cells (NRK-52E) were obtained from National Centre for Cell Science, Pune. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (containing five mM D-glucose; HiMedia, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen) at 37°C with 5% CO_2. The control (normal glucose, NG) group was the serum-free medium of normal glucose DMEM with 5 mM D-glucose, and the HG group was the serum-free medium of HG-DMEM with 30 mM D-glucose. The HG medium was used to mimic the model of diabetes *in-vitro*. The cells were seeded in 6 well plates and, after serum starvation of 12 hrs. was exposed to NG, HG and HG with Cyproheptadine (1 µM) for 48 hrs. Post 48 hrs., the cell lysate was collected for further processing (Fig. 9).

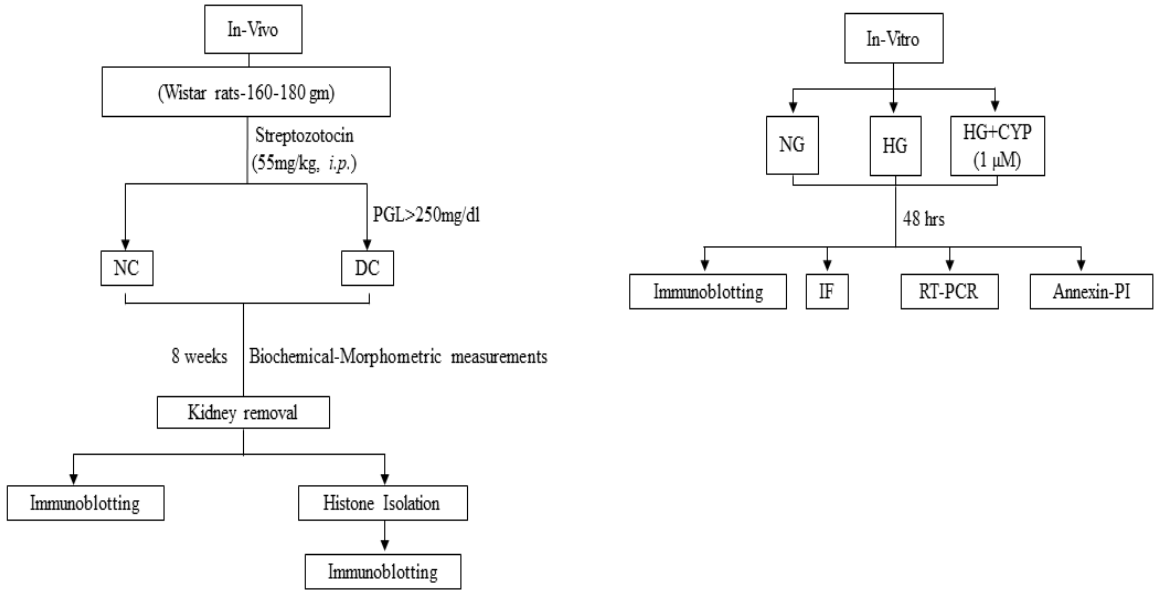


Fig. 9: Schematic representation for the methodology of study 2.

For study 3:

This objective was designed to explore the effect of combination therapy of an ER stress inhibitor (TUDCA) with an AT1R blocker (telmisartan) in preventing the progression of DKD. the animals were divided into 5 groups. (i) Normal control (NC), (ii) Diabetic control (DC), (iii) DC treated with telmisartan (10 mg/kg, *p.o.*), (iv) DC treated with TUDCA (200 mg/kg, *i.p.*) (v) DC treated with telmisartan (10 mg/kg, *p.o.*) and TUDCA (200 mg/kg, *i.p.*). All the treatments continued for 4 weeks. Each group consisted of 6 animals (Fig. 10).

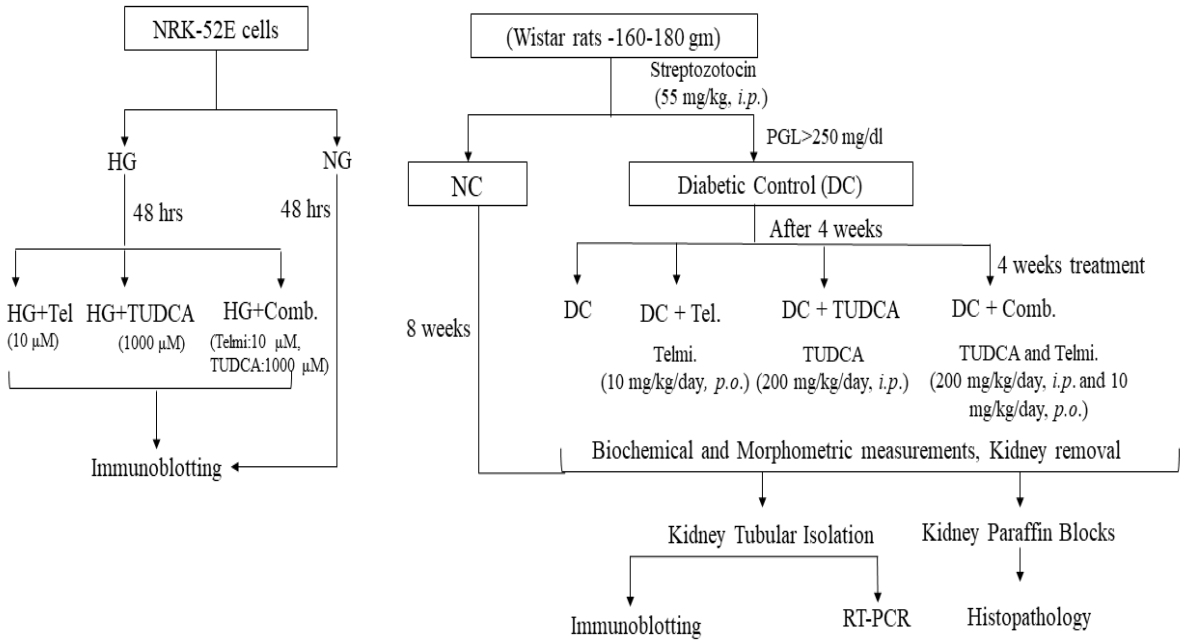


Fig. 10: Schematic representation for the methodology of study 3.

4.4. Biochemical and Morphometric Parameters Evaluation

The biochemical parameters, including plasma glucose, plasma creatinine and BUN was estimated as a marker of renal dysfunction using commercially available Accurex kits. Change in the animals' body weight with time and kidney weight: body weight ratio was also determined to establish the development of T1DM and renal fibrosis.

4.5. Aorta Oxidative Stress Parameters

4.5.1. 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 (Kadakol, Almesh et al., 2017).

4.5.2. Glutathione 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 (Kadakol, Almesh et al., 2017).

4.6. 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% O_2 + 5% CO_2) 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) (Kadakol, A. et al., 2017).

4.7. 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 µmol/mg of protein (Sharma and Gaikwad, 2020).

4.8. Haematoxylin and Eosin Staining

Paraffin blocks of formalin-fixed kidney samples were made followed by sectioning to 5µM thickness for further staining. Morphological alterations were evaluated using Hematoxylin and Eosin (H & E) staining. Briefly, for the procedure the slides were deparaffinized using changes of Xylene followed by rehydration using grades of ethanol (100% to 70%). Post rehydration the slides were stained with haematoxylin and eosin and last mounted using DPX mountant. Each of the staining procedures, 4-5 sections from each kidney and at least 6 kidneys from each group were observed using a Zeiss microscope (AxioVert.A1).

4.9. 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 and kidney tissue were 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) (Kadakol, A. et al., 2017).

4.10. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) Assay

MTT assay was performed using Cyproheptadine in different doses as previously described (Sharma, D. et al., 2019). Briefly, 3000 cells were seeded in 96 well plates and allowed to adhere and grow. Post incubation, the cells were serum-starved for 12 hours. After 12 hours, cells were incubated with different concentrations of Cyproheptadine ranging from 0.1µM-20 µM for 48 hrs. The drugs were aspirated, and the cells were treated with 1mg/ml of MTT for 4 hrs. The formazan crystals were solubilized in DMSO, and the absorbance was taken at 570/630 nm.

4.11. XTT (sodium 3´-[1- (phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy6-nitro) benzene sulfonic acid hydrate)

The viability of the cell against the drugs was evaluated using XTT assay as per the kit protocol (Merck, Darmstadt, Germany). Briefly, the cells were seeded in a 96 well plate in triplicates. Post 24 hrs of incubation the cells were exposed to different doses of telmisartan, TUDCA, and their combination. In the end, 50 µl of XTT solution was added to each well, and absorbance was taken at 450 nm and 670 nm.

4.12. Isolation of Proximal Tubular Region from the Whole Kidney Tissue

Tubules were isolated from the whole kidney using the percoll gradient method as per the earlier laboratory protocol (Sharma, N. et al., 2019). Briefly, the kidneys were minced and digested using collagenase. The lysate was washed thoroughly using PBS followed by the addition of Percoll and high-speed centrifugation at 30,000 RPM for 30 minutes using an ultracentrifuge (Sorvall MX 150+).

4.13. Protein Isolation (cytoplasmic and histone) and Immunoblot Analysis

Rat whole kidney homogenate or cells grown in six-well dishes were lysed in RIPA buffer and sonicated for 3 cycles of 10 seconds. Histones were isolated from the whole kidneys using the acid extraction method, and Immunoblotting was performed as previously described (Chen, S. et al., 2011). Briefly, primary antibodies used in this study were anti-SET7/9 (Cell Signaling Technology, Danvers, MA), TGF-β (Cell Signaling Technology), p-IKKα/β and p- IκBα (Cell Signaling Technology), p-NF-κB and NF-κB (Cell Signaling Technology), anti-H3K4Me1 (Cell Signaling Technology), anti-histone H3 (Cell Signaling Technology), SMAD7, Col4A1, Fibronectin and anti-*β*-actin (Santa Cruz Biotechnology). Secondary antibodies used in this

study were horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (Santa Cruz Biotechnology) or goat anti-mouse immunoglobulin G antibody (Santa Cruz Biotechnology). All the primary antibodies were used in a dilution of 1:1000 (v/v) whereas the dilution used for secondary antibodies was (1:10000). Signals were detected using the ECL reagent (Bio-Rad). The intensity of each band was determined using ImageJ software (version 1.46r; National Institutes of Health, Bethesda, MD).

Table 5 List of antibodies used throughout the study

#	Antibody Name	Dilution	Company
1.	***Primary antibody against:*** SET7/9 (#2813) H3K4Me1 (#5326) H3K9Me2 (#4658) CHOP (#2895) BiP (#3177) p-eif2α (S-51) (#9721) p-NF-κβ (S-536) (#3033) p-IKKα/β p-IKβα IκBα (#4814) p-SMAD2(Ser465/467) (#3108) t-SMAD2 (#5339) p-SMAD2/3 (#8828) α-SMA (#19245) TGF-β (#3709) cleaved PARP (#5625) H3 (#4499)	1:1000 (v/v)	Cell Signaling Technology (Danvers, MA, USA)
2.	***Primary antibody against:*** Fibronectin (sc-8422) SMAD7 (sc-365846) RTN1a (sc-58587) Col1A1 (sc-293182) β-actin (sc-4778)	1:1000 (v/v)	Santa Cruz Biotechnology (Dallas, Texas, USA)
3.	***Secondary antibodies:*** Goat Anti-rabbit IgG (#7074) Horse anti-mouse IgG (#7076) Anti-mouse IgG (Alexa Fluor® 594 Conjugate) #8890	1:20000 (v/v)	Cell Signaling Technology (Danvers, MA, USA)
4.	***Secondary antibodies:*** m-IgG Fc BP-HRP (sc-525409) Mouse anti-rabbit IgG (sc-2357)	1:20000 (v/v)	Santa Cruz Biotechnology (Dallas, Texas, USA)

4.14. Immunofluorescence Assay

For immunofluorescence assay, NRK-52E cells were seeded on coverslips. Post incubation the cells were exposed to normal glucose (5 mM), high glucose (30 mM), and high glucose with Cyproheptadine (1 µM) for a period of 48 hrs. Later, the cells were fixed with 2% paraformaldehyde for 15 minutes and permeabilized using 0.2% Triton X-100. Further, the cells were blocked with 3% BSA in PBS for an hour and incubated with anti-CHOP primary antibody (1:2000 dilution, CST) overnight at 4° C. Next day; the coverslips were washed with ice-cold PBS thrice for 5 minutes each and incubated with Alexa-Fluor488 anti-mouse secondary antibody (1:2000 dilution, CST) for 90 minutes at room temperature. Coverslips were washed thrice from PBS and finally incubated with DAPI for 10 minutes at room temperature. Finally, the coverslips were mounted at the glass slides and visualized using a Zeiss Confocal laser scanning microscope (Li, Z. et al., 2017).

4.15. Annexin-PI Assay

Annexin-PI assay was performed to evaluate the percentage apoptotic cells due to chronic hyperglycaemia and the effect of cyproheptadine in preventing the same. The procedure involved the seeding of cells followed by incubation for 48 hrs. After incubation the culture media was transferred in a falcon tube. The plates were washed with PBS and it was also collected in the same falcon tube. Later, the cells were trypsinized and the lysate was put in the same falcon tube having culture media and PBS. The whole mixture was centrifuged. The pellet was kept on ice and washed thrice with ice cold PBS. The supernatant was discarded and binding buffer was added. This was followed by addition of annexin V and incubation of 15 minutes and subsequent addition of PI and incubation for 10 minutes in dark at 37^0C. The cells were analysed using Beckman coulter Flow cytometer and the data was analysed using CytExpert software.

4.16. RNA Isolation and Real-Time Polymerase Chain Reaction (RT-PCR)

RNA isolation and RT-PCR were performed as described previously (Chen, S. et al., 2011). Briefly, RNA was isolated from the whole kidney homogenate, kidney tubules and NRK-52E cells using the Trizol method followed by cDNA synthesis using GeneSure H- Minus First Strand cDNA Synthesis Kit. Further, the cDNA was reverse transcribed, and RT-PCR was performed using the iTaq Universal SYBR Green Supermix (Bio-Rad, USA). Relative expression of each gene of interest was evaluated on LightCycler® 96 Real-Time PCR System (Roche) using LightCycler Software (Roche). The abundance of targeted mRNA was normalized against GAPDH or 18s mRNA.

Table 6: List of primer sequences utilized for qRT-PCR.

Gene Name	Primer sequences of qRT-PCR
Casp7	Forward:5'-ACCGCTCCACCATCATCTCA-3' Reverse:5'- CGGACATCCATACCTGTCGCT-3'
Tgfb1	Forward: 5'-CTGCTGACCCCCACTGATAC-3' Reverse: 5'-AGCCCTGTATTCCGTCTCCT-3'
Col1a1	Forward: 5'-TGGCAACCTCAAGAAGTCCC-3' Reverse: 5'-ACAAGCGTGCTGTAGGTGAA-3'
SMAD7	Forward: 5′-GGGTTTACAACCGCAGCAGT-3′ Reverse: 5′-GCCTTGATGGAGAAACCAGG-3′
CHOP	Forward: 5'-CCTGAGGAGAGAGTGTTCCAG-3' Reverse: 5'-TCAAAGGCGAAAGGCAGAGA-3'

4.17. *Statistical Analysis*

Statistical analysis was performed using GraphPad Prism (8.0.2). To evaluate the significant difference between groups, a t-test was used when two groups were compared and a one-way analysis of variance (ANOVA) while comparing more than two groups. Tukey's test was used for multiple comparisons. Statistical significance was considered when $p < 0.05$. All the results were expressed as mean ± SEM (Goru, Santosh Kumar et al., 2017).

Chapter 5

Results

5. Results

5.1. Inhibition of endoplasmic reticulum stress combined with activation of angiotensin-converting enzyme 2 prevented endothelial dysfunction

5.1.1. Diminazene aceturate and TUDCA combination therapy normalized the blood glucose levels

T1DM 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. 11a).

5.1.2. 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. (11b; 11c). However, the combination therapy brought a significant reduction in the MDA levels as compared to monotherapies.

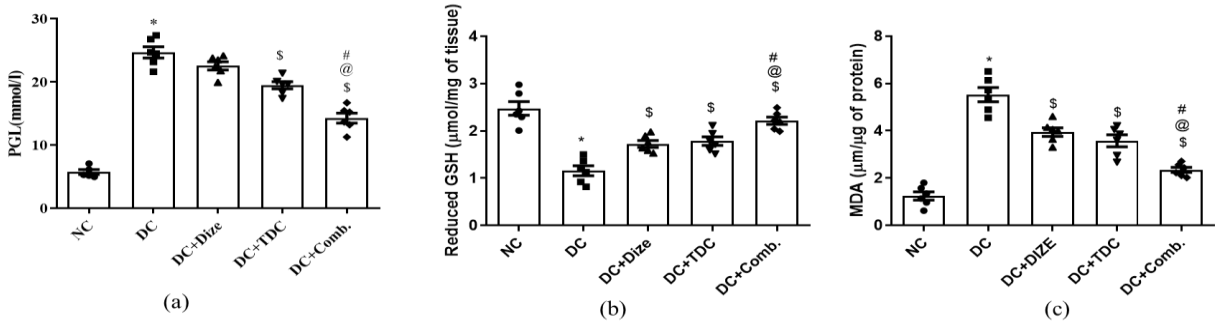


Fig. 11: Effect of different treatments on plasma glucose levels and aortic oxidative stress parameters.

*All the values are represented as mean ± 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.

5.1.3. 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. 12). The pD_2 values and Emax are provided in Table 7.

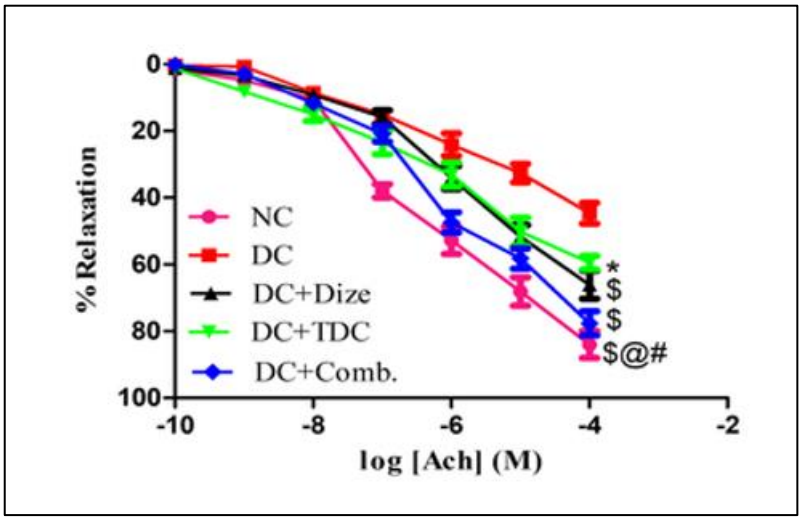


Fig. 12: Concentration-dependent relaxation of acetylcholine on aortic rings (with intact endothelium) pre-contracted with phenylephrine obtained from different treatment groups.

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

Table 7: 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).

	Acetylcholine	
	pD_2	**E_{max} (% 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$@#

5.1.4. 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. 13).

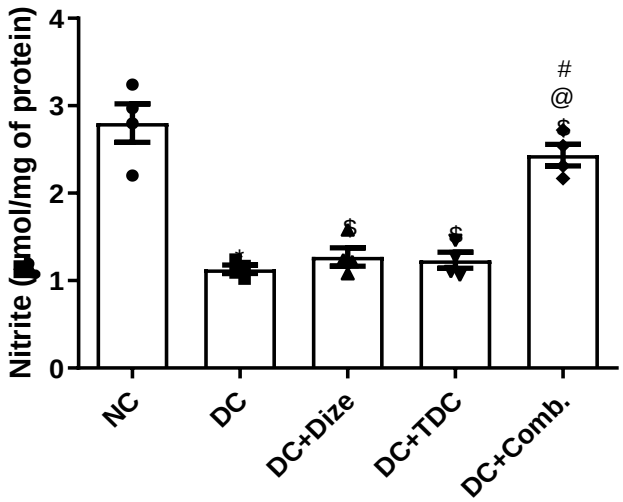


Fig. 13: **Effect of different treatments for 4 weeks on aortic nitrite levels.**
Aortic nitrite levels in different groups. All the values are represented as mean ± SEM; n=6 (p<0.05; ** vs NC; $ vs DC; @ vs DC+Dize; # vs DC+TDC).

5.1.5. 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 colour 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. 14).

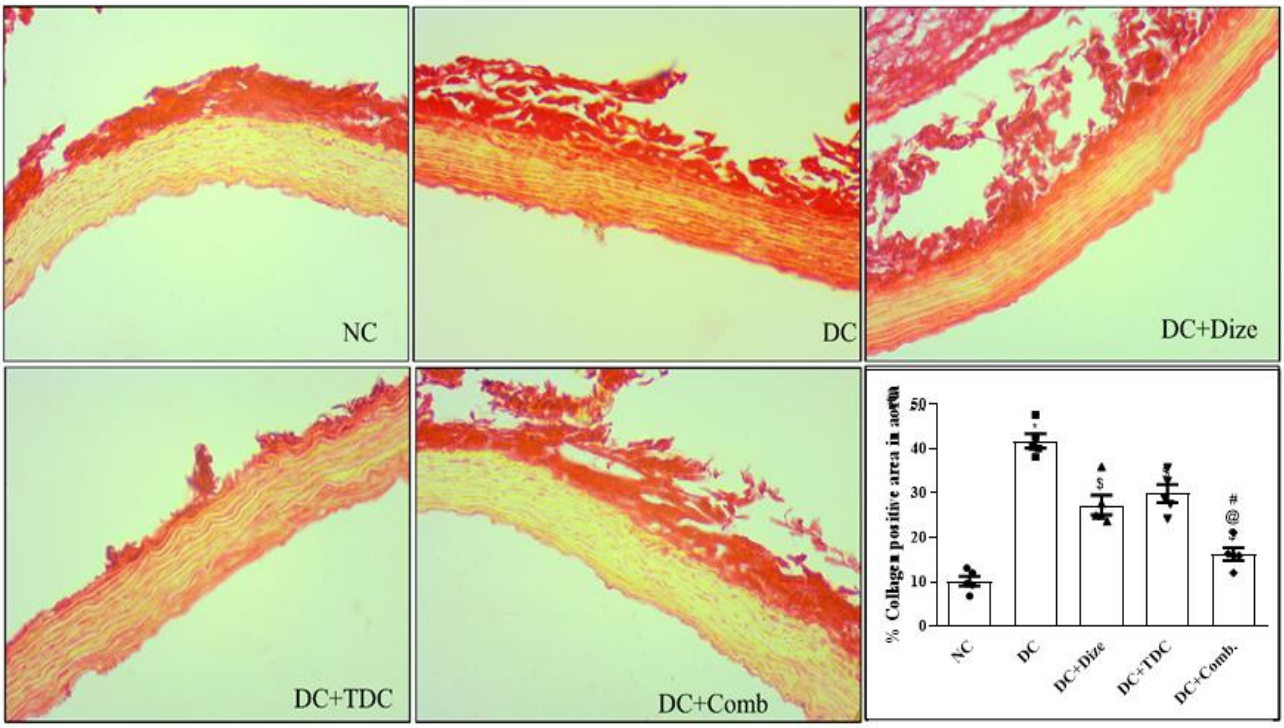


Fig. 14: Light microscopic pictures illustrating the Picrosirius red (PSR) staining for collagen deposition (400× magnification) and quantification of (B) % positive area of collagen.

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

5.2. Epigenetic regulation of ER stress in the diabetic kidney and high glucose treated NRK-52E cells

5.2.1. Morphometric and biochemical changes in the animals with DKD

Post 48 hrs. of STZ administration, all the animals developed hyperglycemia with plasma glucose levels < 21 mmol/l. Markers of the kidney damage including BUN and plasma creatinine were also on the higher side in the diabetic animals compared to the normal control (Table 8). In diabetic animals, there was a drastic reduction in body weight after 8 weeks of diabetes. Also, the kidney weight of the diabetic animals was significantly higher along with the kidney weight and body weight ratio (Table 8). All the above parameters indicate the presence of progressive kidney damage.

Table 8: Biochemical and Morphometric parameters of the normal and diabetic animals.

Biochemical Parameters				Morphometric Parameters		
Group	PGL (mmol/l)	PCr (mg/dl)	BUN (mg/dl)	BW (gm)	KW (gm)	(KW/BW) × 100
NC	5.16 ± 0.49	1.1 ± 0.12	21.36 ± 1.2	280 ± 12	0.74 ± 0.04	0.26± 0.016
DC	21.14 ± 1.12*	2.1 ± 0.17*	48.19 ± 3.1*	160 ± 6*	1.0 ± 0.03*	0.62 ± 0.038*

All the values are represented as mean ± SEM (n = 8). ***Abbreviations:*** *PGL, plasma glucose level; PCr, plasma creatinine; BUN, blood urea nitrogen; NC, normal control; DC diabetic control; BW, body weight; KW, kidney weight. *P< 0.05 vs. NC.*

5.2.2. Cyproheptadine dose determined using cell viability assay

Cell viability assay using MTT was used to determine the cytotoxic potential. It helps in deciding the dose of the drug to be used in the study. In this assay, Cyproheptadine was used starting from the initial dose of 0.1µM up to 20µM. Cell viability of more than 75% was observed in 0.1, 0.5, and 1µM Cyproheptadine (Fig. 15). Thus, we chose the dose of 1 µM for our study, since 1 µM was reported to be the IC50 value of the Cyproheptadine against SET7/9.

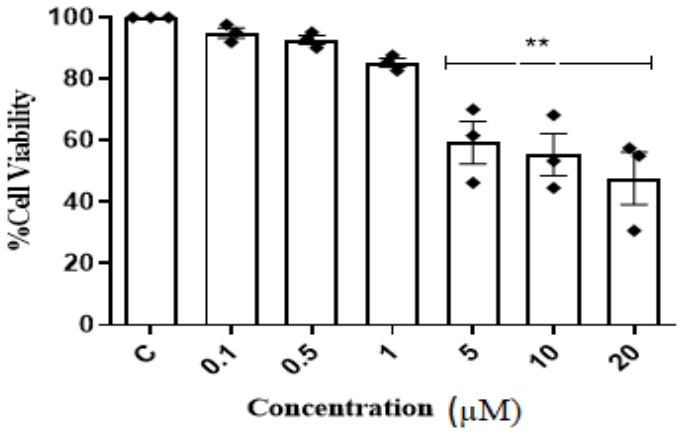


Fig. 15: Cell viability assay using MTT to determine the dose of the drug to be used for the study. To determine the cell viability cells were incubated with cyproheptadine at different doses from 0.1 µM up to 20 µM. All the values are represented as mean ± SEM (n=3). One-way ANOVA with Tukey's multiple comparisons test, where (**) $p<0.05$ vs. Control (C).

5.2.3. Increase in the expression of SET7/9 and H3K4Me1 and decrease in H3K9Me2 in the diabetic kidney and NRK-52E cells under hyperglycemic condition

Hyperglycemia is known to cause epigenetic changes in the kidney. Several HMTs such as Enhancer of zeste homolog 2 (Ezh2), Suppressor of Variegation 3-9 Homolog 1 (SUV39H1), G9a, SET7/9 etc. are reported to regulate the progression of kidney diseases (Yu and Zhuang, 2019). We found a significant increase in the level of SET7/9 in the kidney of diabetic animals and the NRK-52E cells incubated with high glucose (30 mM) for 48 hrs. This indicates the role of hyperglycemia in increasing the histone methylation marks (H3K4Me1) and adding to the progression of DKD. We observed higher expression of H3K4Me1 in the hyperglycemic NRK-52E cells as well as diabetic kidney compared to the respective normal controls which is the active chromatin mark and activates the genes responsible for the progression of DKD (Fig. 16a, 16b). A decrease in H3K9Me2 was also found in the histones isolated from kidneys of diabetic rats indicating a decrease in repressive genes again promoting DKD advancement (Fig.

c). This increase in the active chromatin marks and decrease in the repressive chromatin marks lead to the activation of the fibrotic and inflammatory genes.

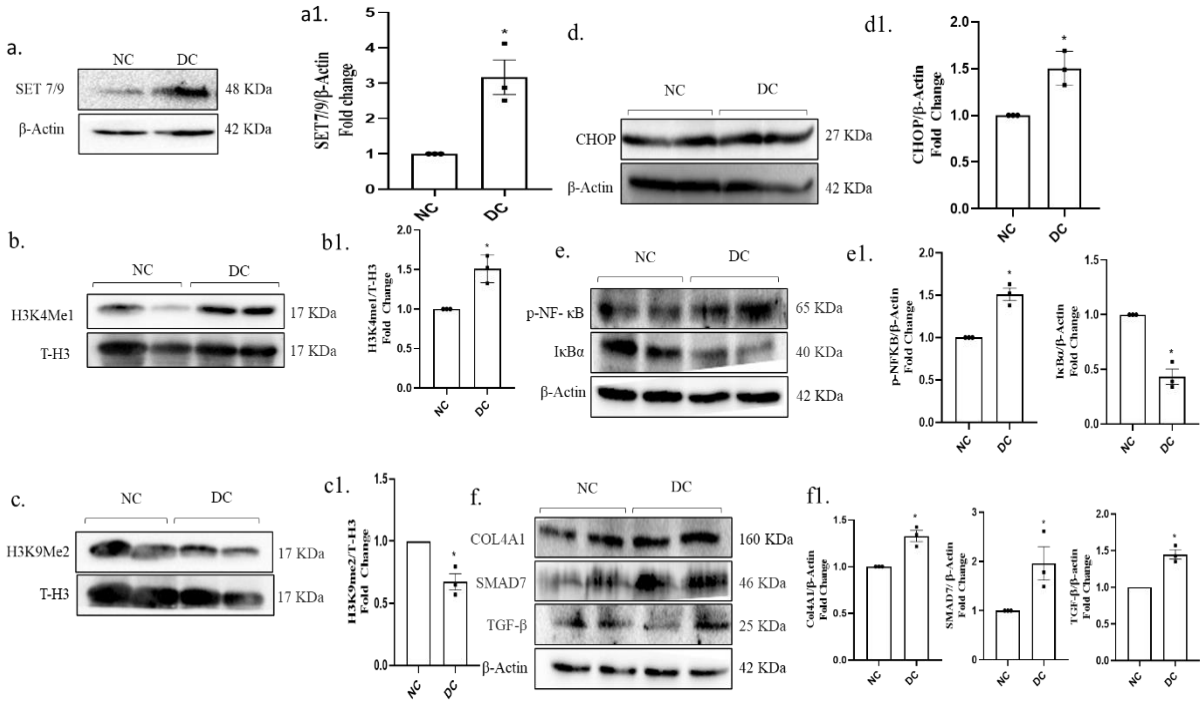


Fig. 16: Diabetic kidney disease increases the protein expression of SET7/9, H3K4Me1 and, pro-inflammatory and profibrotic markers and decreases the H3K9Me2 expression in the kidney of rats. *a-f) Representatives immunoblot images and scattered-bar plots, (a1-f1) depicted fold change in protein expressions of SET7/9, H3K4Me1, H3K9Me2, CHOP, p-NF-κB(S-536), IκBα, p-IκBα, TGF-β, Smad7, Col4A1 when compared with NC rats' kidney, respectively. β-actin and TH3 was used as a loading control to normalize protein amount. All the values are represented as mean ± SEM; n=3. [(*) $p < 0.05$ vs NC]. t-test was used to evaluate the significance level amongst the groups.*

5.2.4. Incubation with Cyproheptadine reduced the expression of H3K4Me1 in NRK-52E cells

Previous studies have established the association between increased expression of H3K4Me1 and transcriptional activation of TGF-β signaling (Sasaki et al., 2016). SET7/9 is known to catalyze the activation of H3K4Me1. So, we evaluated the effect of Cyproheptadine on the expression of H3K4Me1 in NRK-52E cells. The enhancement in the active chromatin mark (H3K4Me1) due to persistent hyperglycemia increases the inflammatory and fibrotic signaling. We found that Cyproheptadine treatment significantly suppressed the expression of H3K4Me1 (Fig. 17a).

5.2.5. Cyproheptadine treatment curbed the NF-κB signaling pathway activation in NRK-52E cells

Persistent hyperglycemia and development of DKD in the animals could be attributed to activation of the pro-inflammatory NF-κB signaling pathway (Foresto-Neto et al., 2020). We also observed an enhanced level of p-NF-κB and a reduced level of IκBα in the kidneys of the diabetic animals (Fig. 16e). Furthermore, NRK-52E cells upon exposure to high-glucose for 48 hours also showed elevated levels of p-NF-κB and p- IKKα/β and p-IκBα (Fig. 17b). The cells when treated with Cyproheptadine, showed marked inhibition of the NF-κB signaling as observed by reduced p-NF-κB and p-IKKα/β and p-IκBα (Fig. 17b). These results indicate that treatment with Cyproheptadine reduces inflammation.

5.2.6. Cyproheptadine exposure significantly abrogated the profibrotic TGF-β signaling in NRK-52E cells

TGF-β signaling is central to the development of renal fibrosis, which is the outcome of most chronic kidney diseases including DKD (Sasaki et al., 2016). In the kidney of the diabetic animals, TGF-β signaling was dysregulated as evidenced by the increased TGF-β, alpha 1 chain of type IV collagen (Col4A1) and decreased SMAD7 levels (Fig. 16f). A significant increase in the levels of fibronectin and collagen was also noted. More importantly, Cyproheptadine significantly attenuated the TGF-β signaling in the NRK-52E cells which were shown by reduced TGF-β, pSMAD2 expression and enhanced SMAD7 levels (Fig. 17c). Increased mRNA expression of SMAD7 in Cyproheptadine treated NRK-52E cells also indicates the reduction in fibrosis (Fig. 17d).

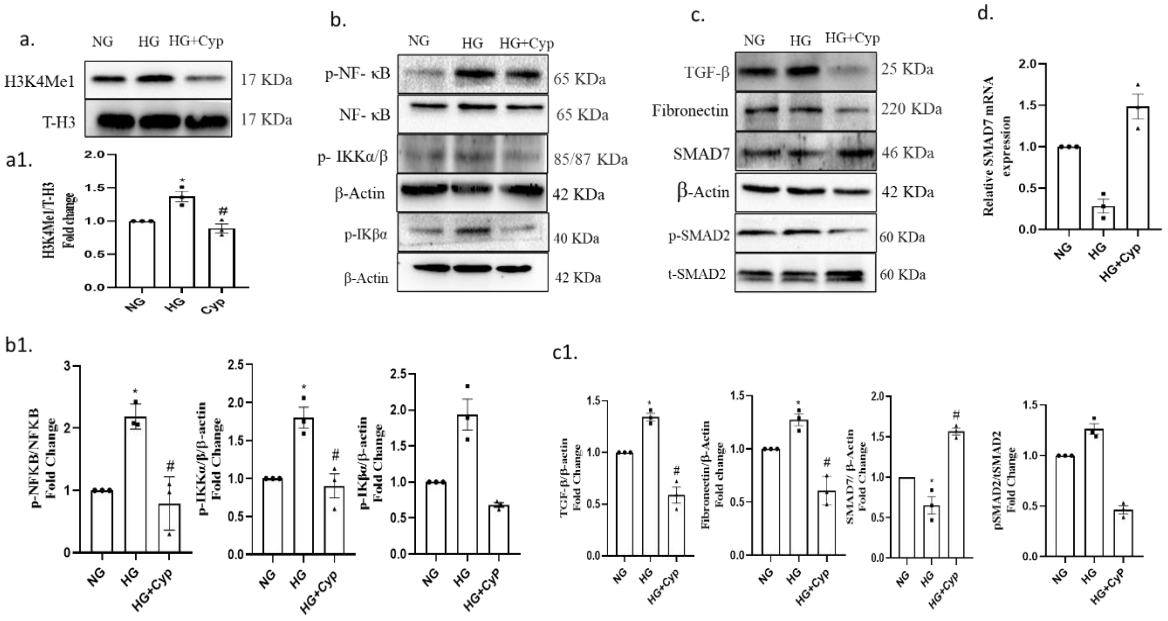


Fig. 17: Cyproheptadine treatment normalized histone methylation and ameliorated inflammation and fibrosis. *a) Representative western blot images for protein expressions of H3K4Me1 normalized against TH3 in NRK-52E cell lysates. b-c) Represents, immunoblots for inflammatory [p-NF-κB (S-536, p-IKβα and p-IKKα/β] and fibrotic (TGF-β, p-SMAD2 and SMAD7) markers. Respective β-actin, NF-κB or p-SMAD2 were used as a loading control. a1-c1) depicted fold change in protein expressions of the immunoblots in Fig. a-c. Fig. 17d. Represents fold change in the SMAD7 mRNA expression normalized using 18S RNA. All the values are represented as mean ± SEM (n=3). One-way ANOVA with Tukey's multiple comparisons test, where (*) p < 0.05 vs. NG; (#) p < 0.05 vs. HG was used.*

5.2.7. Cyproheptadine treatment reduced the expression of CHOP and p-eif2α in NRK-52E cells

ER stress has emerged as a decisive factor in the progression of the DKD (Chen, J. et al., 2014). The current study also found a significant increase in the ER stress markers such as phospho-eukaryotic initiation factor 2 α (p-eif2α) and C/EBP homologous protein (CHOP) *in-vivo* (Fig. 16d) and *in-vitro* (Fig. 18a-c). Interestingly, we observed that Cyproheptadine significantly ameliorated the ER stress compared to the hyperglycemic group, as observed by the reduced expression of both p-eif2α and CHOP. Similarly, during immunofluorescence assay, the fluorescence intensity of CHOP was significantly higher in the cells incubated with high glucose (30 mM). However, the intensity considerably diminished in the co-incubated cells with high glucose and Cyproheptadine. A significant reduction in the mRNA levels of CHOP was also observed in Cyproheptadine-treated NRK-52E cells (Fig. 18b). These results indicate

that Cyproheptadine treatment significantly attenuates the ER stress and ensuing kidney disease progression possibly by inhibiting the SET7/9.

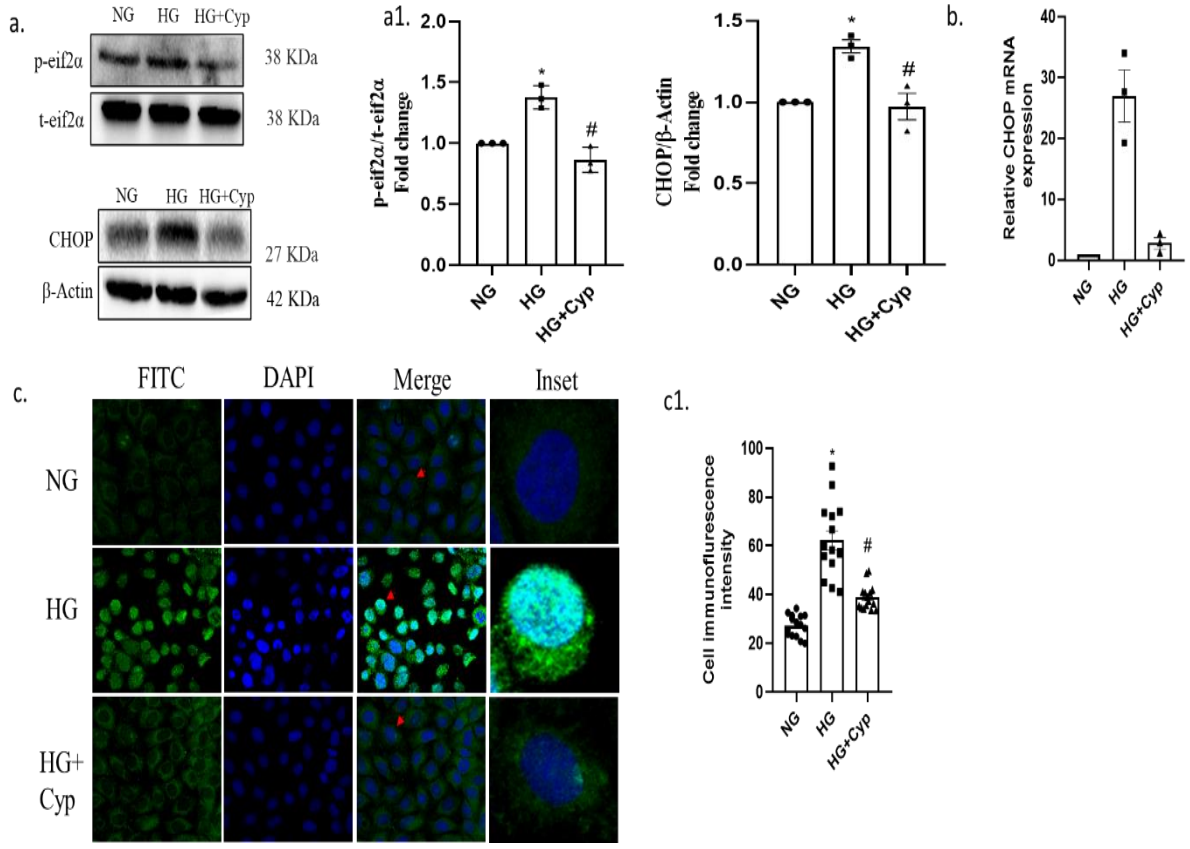


Fig. 18: Incubation with Cyproheptadine lead to reduced expression of CHOP and p-eif2α in NRK-52E cells. *a) Representative western blot images for protein expressions of p-eif2α and CHOP. t-eif2α and β-actin were used as loading controls. a1) depicted fold change in protein expressions of the immunoblots of p-eif2α and CHOP. b) Represents the change in relative mRNA expression of CHOP. c) Immunofluorescence of CHOP exposed to normal glucose, high glucose and high glucose with Cyproheptadine. CHOP is shown by green fluorescence, and cell nuclei stained with 4`,6-diamidino-2-phenylindole by blue fluorescence. All the values are represented as mean ± SEM (n=3). c1) depicts the change in the relative quantification of CHOP by cells based on their fluorescent intensity. One-way ANOVA with Tukey's multiple comparisons test was used for statistical analysis, where (*) $p < 0.05$ vs. NG; (#) $p < 0.05$ vs. HG.*

5.2.8. Cyproheptadine treatment reduced the hyperglycemia induced cell apoptosis in NRK-52E cells

Cells incubated with HG showed considerable elevation in apoptosis when compared to the those incubated with NG. Interestingly, Cyproheptadine treatment of the cells reduced the apoptosis significantly in comparison to the HG group (p<0.05) (Fig. 19).

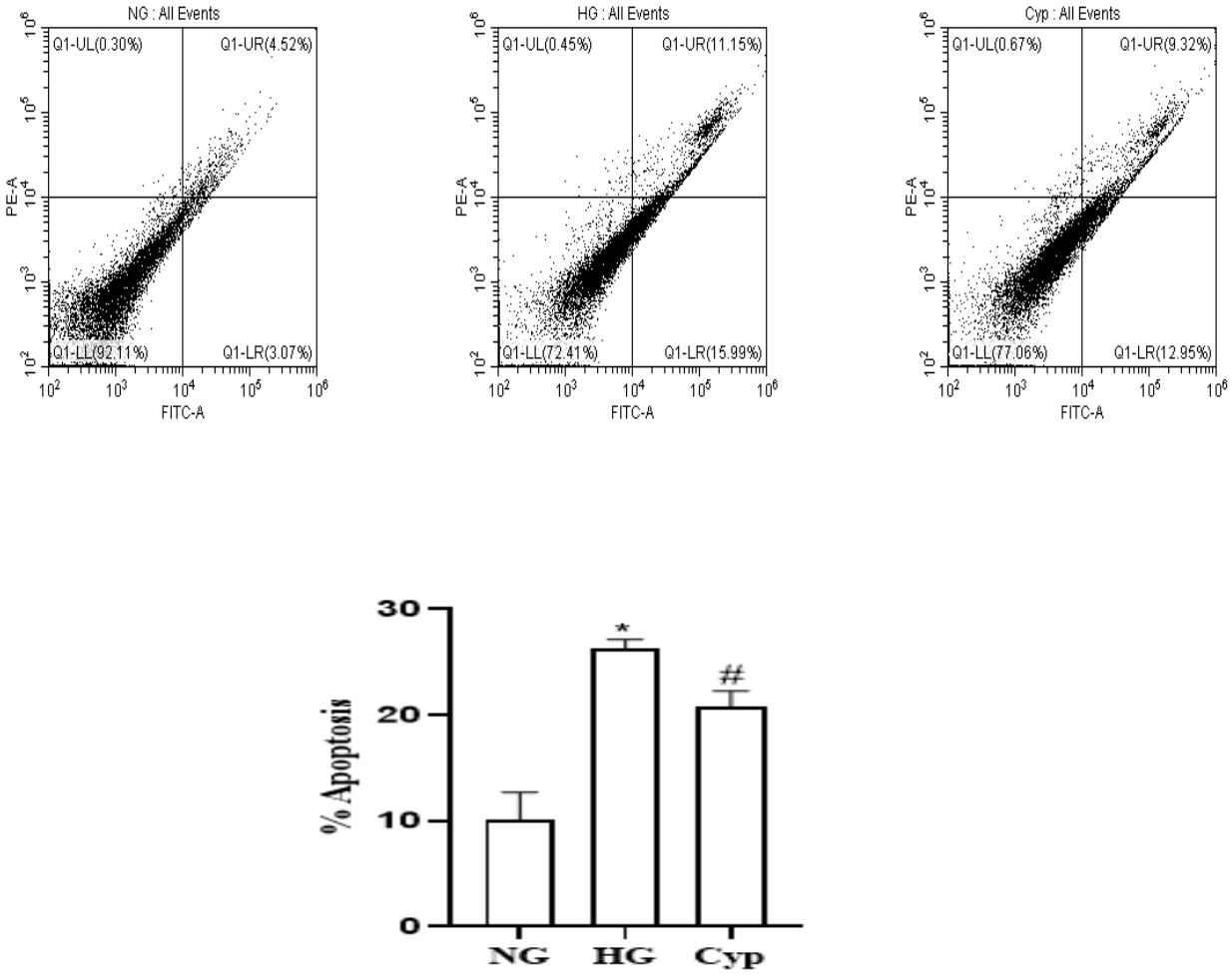


Fig. 19: Cyproheptadine treatment significantly reduced percentage apoptotic cells. *In the graph Q2 shows the cells in early apoptotic stage and Q3 the late apoptotic stage of the cells. Percentage apoptotic cells is the sum of Q2 and Q3. The percentage apoptotic cells have significantly increased in HG group when compared to NG group which reduced on treatment with Cyproheptadine. All the values are represented as mean ± SEM (n=3). One-way ANOVA with Tukey's multiple comparisons test was used for statistical analysis, where (*) p < 0.05 vs. NG; (#) p < 0.05 vs. HG.*

5.3. Role of the ER stress inhibition combined with AT1R blockade in preventing the progression of DKD

5.3.1. Effect of the treatment of TUDCA, telmisartan, and their combination on the morphometry of the animals

After 8 weeks of the diabetes induction, the diseased control group exhibited a significant decline in body weight as compared to the normal control animals. Treatment with monotherapies as well as combination therapy prevented the loss in weight of the animals. However, the combination therapy prevented the loss in the body weight and increase in kidney weight of the animals significantly as compared to the diabetic control and the respective monotherapies. Thus, there was a significant reduction in the relative kidney weight suggesting that there is a reduction in renal hypertrophy by the combination therapy (Table 9).

Table 9: Effect of different treatments on the body weight, kidney weight, and relative kidney weight (KW/BW ∗ 100) of the treated animals.

Morphometric Parameters			
Group	BW (gm)	KW (gm)	(KW/BW) × 100
NC	264 ± 17	1.20 ± 0.09	0.45 ± 0.02
DC	154 ± 08*	1.49 ± 0.06*	0.96 ± 0.03*
DC+ Telmi	181 ± 07$^{\#}$	1.06 ± 0.07$^{\#}$	0.58 ± 0.02 $^{\#}$
DC + TDC	172 ± 07$^{\#}$	1.19 ± 0.03$^{\#}$	0.69 ± 0.04 $^{\#}$
DC + Comb.	197 ± 12$^{\#@}$	0.91 ± 0.06 $^{\#@}$	0.46 ± 0.02$^{\#@}$

All the values are represented as mean ± SEM (n = 6) [(*) p < 0.05 vs NC; (#) p < 0.05 vs DC; (@) p < 0.05 vs monotherapies].

BW: Body weight; KW: Kidney weight; NC: Normal Control; DC: Diabetic control; DC+Telmi: Diabetic control treated with telmisartan; DC+TDC: Diabetic control treated with tauroursodeoxycholic acid; DC+ Comb.: Diabetic control treated with a combination of telmisartan and tauroursodeoxycholic acid.

5.3.2. TUDCA and Telmisartan combination prevented the hyperglycemia and decline in renal function associated with DKD

At the end of the 8 weeks, the animals in the diabetic control group exhibited a steep increase in the plasma glucose levels along with a significant decline in kidney function which was depicted by an increase in the blood urea nitrogen and plasma creatinine (Fig. 20a). Telmisartan and TUDCA both the monotherapies reduced the plasma glucose significantly ($p<0.05$) when compared to the diabetic control group. This could be because TUDCA is known to prevent β-cell apoptosis along with the degradation of insulin-degrading enzymes in the liver. Similarly, they also led to a significant decline in the levels of plasma creatinine and blood urea nitrogen (Fig. 20b and 20c). Furthermore, we observed that the reduction in plasma glucose, creatinine, and BUN was more pronounced in the combination group ($p<0.05$) in comparison to the monotherapies (Fig. 20a-20c).

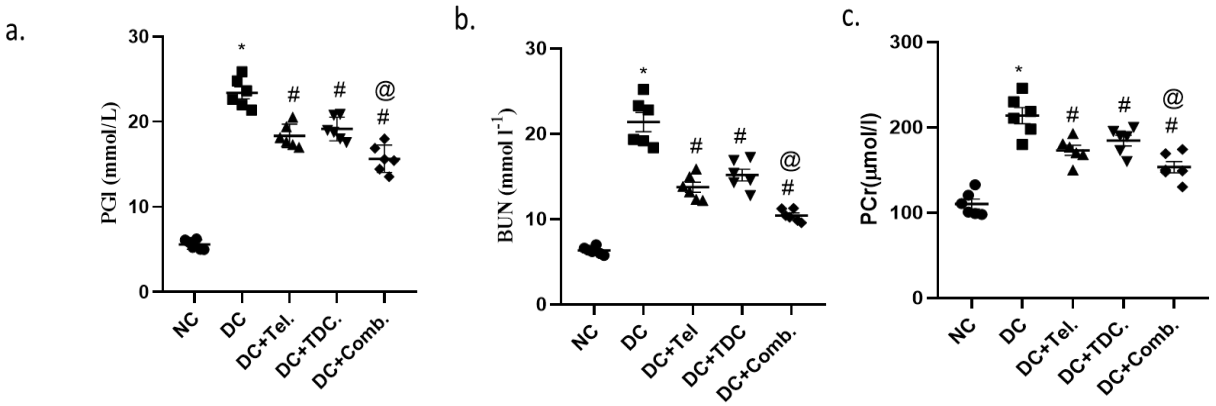


Fig. 20a-20c: Plasma biochemistry. *Plasma biochemistry was carried out at the end of 8 weeks using commercially available kits. Scattered plots, 20a)–20c) displayed plasma levels of glucose (PGl), blood urea nitrogen (BUN), and creatinine (PCr), respectively. All the values are represented as mean ± SEM; n = 6. [(*) p < 0.05 vs NC; (#) p < 0.05 vs DC; (@) p < 0.05 vs monotherapies].*

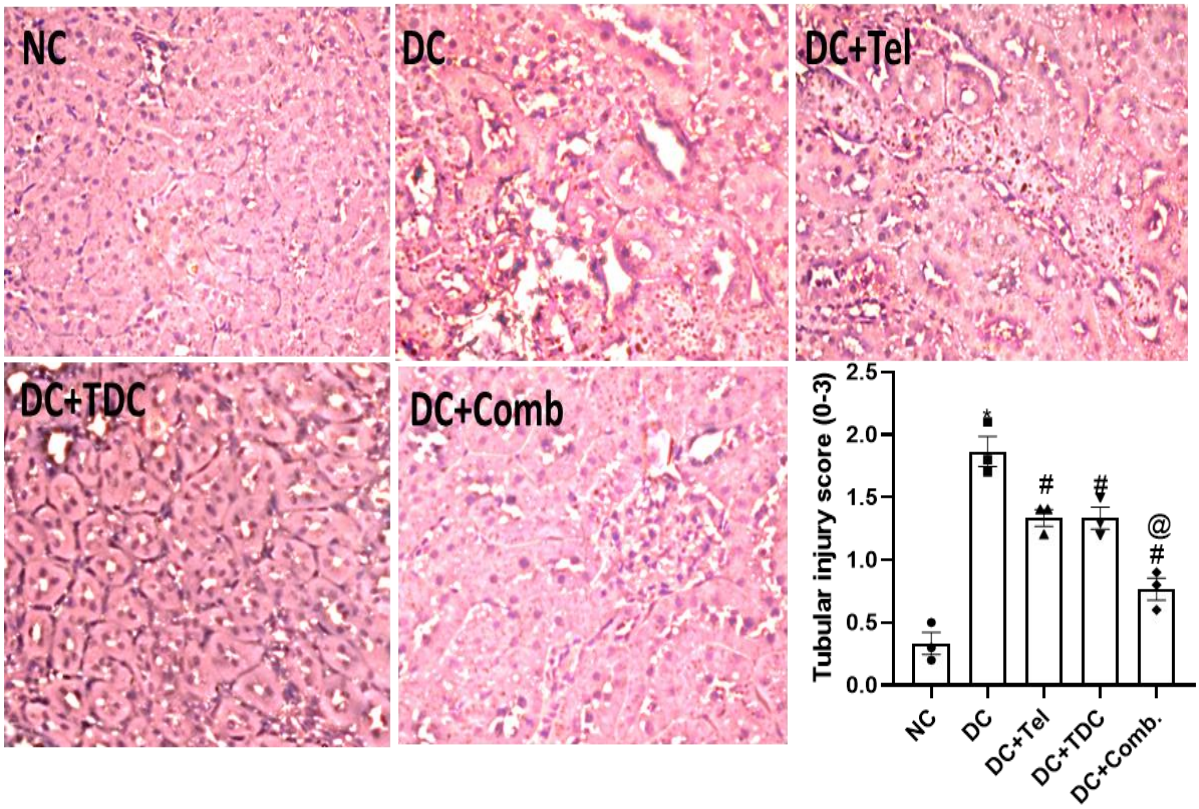


Fig. 20d. *H & E-stained images of the cortical region of kidney transverse sections at 400× magnification. 4–to 5 images from every kidney section and 6 kidneys from each group were evaluated by a blinded observer for tubular necrosis. The tubular necrosis in each image was given a score between (0-and 3). NC: Normal Control; DC: Diabetic control; DC+Telmi: Diabetic control treated with telmisartan; DC+TDC: Diabetic control treated with tauroursodeoxycholic acid; DC+ Comb.:Diabetic control treated with a combination of telmisartan and tauroursodeoxycholic acid.*

5.3.3. Effect of the combined treatment of TUDCA and telmisartan on the renal morphology and tubulointerstitial fibrosis associated with DKD

To evaluate the extent of morphological changes brought about by DKD we performed H and E staining. We observed extensive tubular injury in the kidney of the diabetic rats. However, upon treatment with telmisartan, TUDCA, and their combination we found that the tubular injury was significantly minimized in the treated groups when compared to the diabetic group (Fig. 20d). Picrosirius red staining is used to identify collagen deposition.

In this study, we found that PSR stained slides on microscopic analysis revealed a marked increase in the tubulointerstitial fibrosis in the kidney of diabetic rats. Both the telmisartan and TUDCA treatment produced a marked reduction in the deposition of collagen in the interstitium of the tubules as evidenced by the reduced Sirius stain (Fig. 21). Moreover, the reduction

brought about by the combination therapy was more prominent ($p<0.05$) in comparison to telmisartan or TUDCA alone.

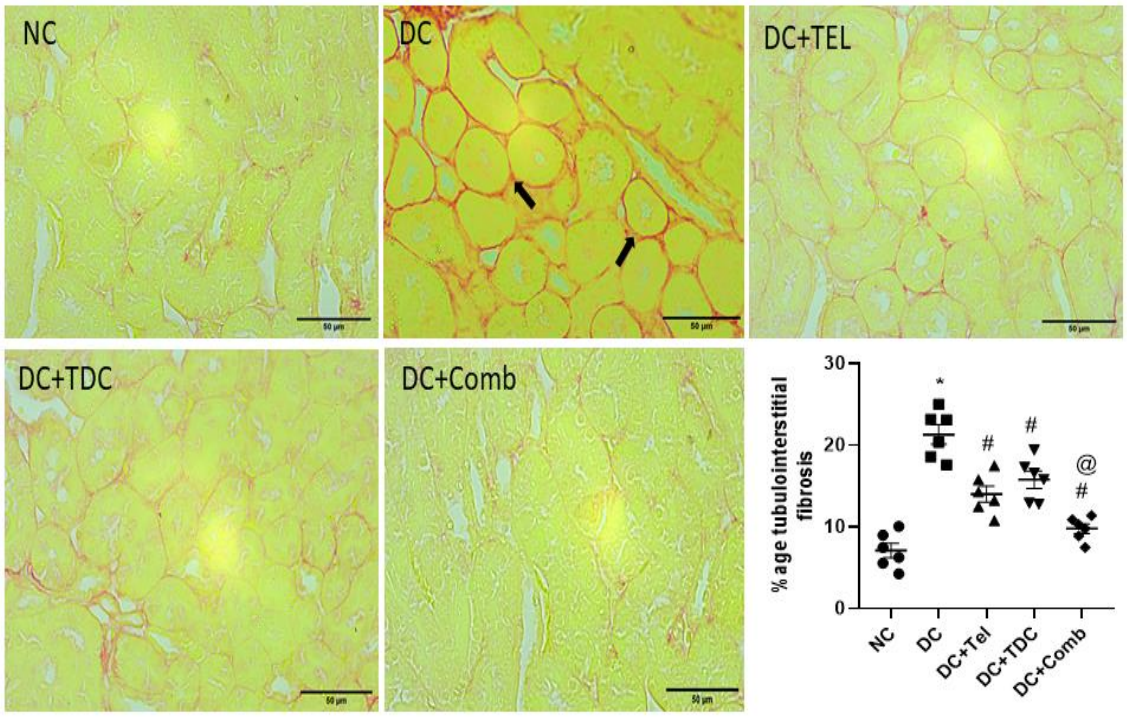


Fig. 21. Effect of different treatments on diabetes-induced tubulointerstitial fibrosis.

*Light microscopic pictures illustrate the Picrosirius red (PSR) staining for collagen deposition (400× magnification and scale bar- 50 µM) representing tubulointerstitial fibrosis. All values are represented as means ± SEM; n=6 ($p<0.05$; * vs NC; # vs DC; @ vs monotherapies].*

5.3.4. TUDCA per se and in combination with telmisartan abrogated the ER stress in the diabetic kidney tubules and NRK-52E cells

Both the tubules isolated from the kidney of diabetic rats and the NRK-52E cells exposed to high glucose showed an elevated ER stress which was measurable at both mRNA and protein levels. We found significantly higher expression of BiP, p-eif2α, and CHOP as compared to respective normal control samples both *in-vivo* and *in-vitro* indicating an increase in the ER stress and the resulting activation of the UPR pathway (Fig. 22a and 22b). TUDCA as well Telmisartan both reduced the ER stress and restored the UPR signaling as can be seen by the reduced expression of BiP and CHOP. Moreover, the combination of these two drugs was significantly potent ($p<0.05$) in reducing the ER stress with respect to the monotherapies (Fig.

22a and 22b). The *in-vitro* dose of the telmisartan, TUDCA, and the combination was based on the cell viability assay result (Fig. 23).

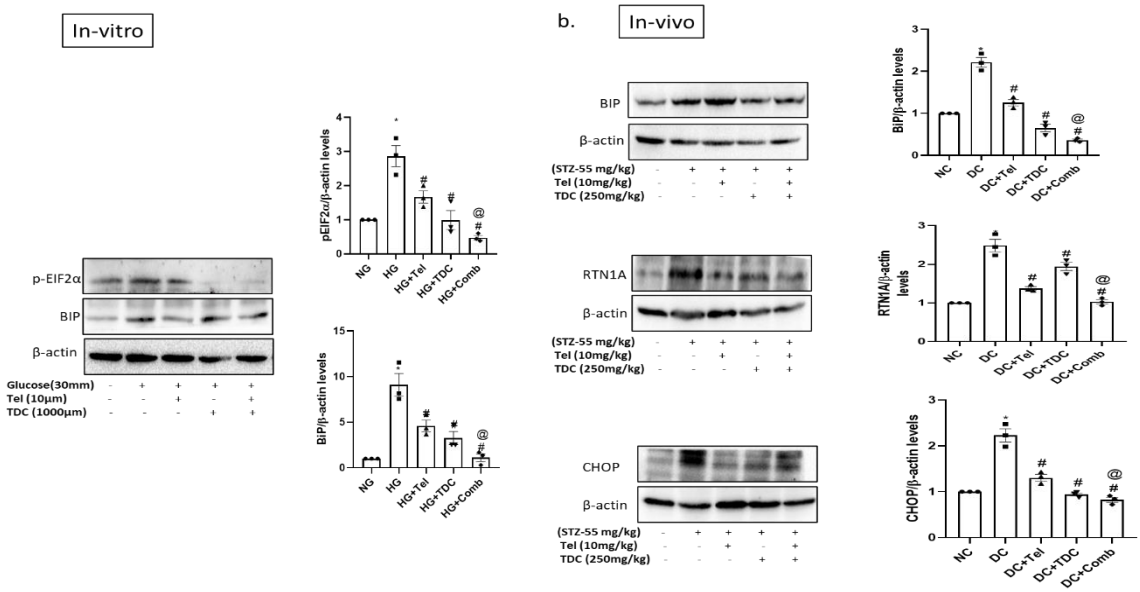


Fig. 22. Effect of the telmisartan and TUDCA monotherapies and combination therapy on ER stress in the kidney tubules of diabetic rats and NRK-52E cells incubated with high glucose. 22a. *Representative immunoblots and fold change in the expression of BiP and p-eif2α in-vitro.* ***22b.*** *Representative immunoblots and fold change in the expression of BiP, RTN1a and CHOP in in-vivo tubular samples. All the values are represented as mean ± SEM; n = 3. [(*) p < 0.05 vs NC; (#) p < 0.05 vs DC; (@) p < 0.05 vs monotherapies].*

NG: Normal glucose; HG: High glucose; NC: Normal Control; DC: Diabetic control; DC+Telmi: Diabetic control treated with telmisartan; DC+TDC: Diabetic control treated with tauroursodeoxycholic acid; DC+ Comb.: Diabetic control treated with a combination of telmisartan and tauroursodeoxycholic acid

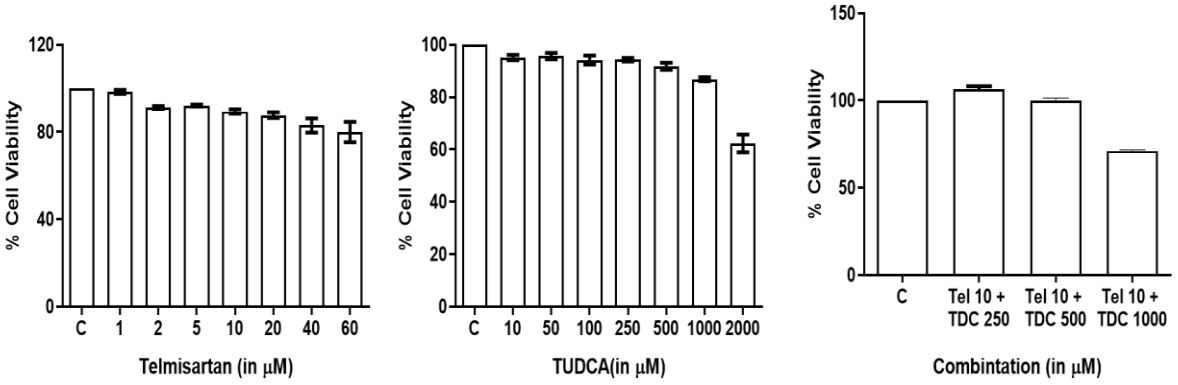


Fig. 23: Cell viability assay to determine the dose of Telmisartan, TUDCA and their combination using XTT.

5.3.5. The combination of TUDCA and telmisartan significantly attenuated the pro-apoptotic signaling pathway in DKD and NRK-52E cells exposed to high glucose

Kidney tubules from diabetic animals and the NRK-52E cells incubated with high glucose (30 mM) exhibited enhanced apoptosis as can be seen in the increased apoptotic markers such as cleaved-PARP and cleaved-caspase 3 at protein level (Fig. 24a and 24b) and caspase 7 and 8 at mRNA level (Fig. 24c). Upon treatment with telmisartan, TUDCA, and their combination there was a marked decrease in apoptosis ($p<0.05$) indicated by the reduced protein and mRNA expression of the different apoptotic markers.

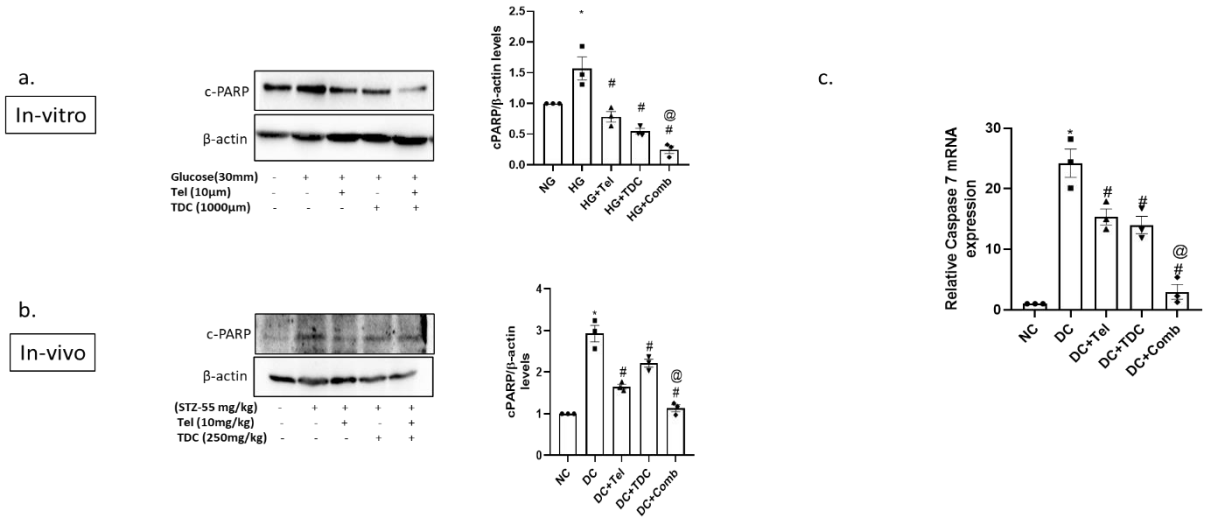


Fig. 24. Treatment of telmisartan and TUDCA *per se* and in combination reduced the apoptosis significantly both *in-vitro* and *in-vivo* at mRNA and protein levels. 24a. Representative immunoblot and fold change in the expression of c-PARP in the NRK-52E cells. **24b.** Representative immunoblot and fold change in the expression of c-PARP in the kidney tubules. **24c.** Represents the fold change in the expression of the caspase 7 mRNA in the isolated kidney tubules with 18S as the internal control. All the values are represented as mean ± SEM; n = 3. [(*) $p < 0.05$ vs NC; (#) $p < 0.05$ vs DC; (@) $p < 0.05$ vs monotherapies].

5.3.6. Treatment with the combination of telmisartan and TUDCA reduced the profibrotic signaling

Fibrosis is the ultimate endpoint in the pathophysiology of DKD (Moeller et al., 2022). Here also, we observed an augmentation in the mRNA and protein expression of TGF-β, p-SMAD2/3, COL1A1 and attenuation in the protein (Fig. 25a and 25b) and mRNA expression of SMAD7 both *in-vitro* and *in-vivo* (Fig. 25c). Moreover, we found that the profibrotic

pathways were significantly halted by the treatment of the telmisartan and TUDCA. Furthermore, the reduction brought about by the combination of the drugs was significantly better than TUDCA for all the profibrotic markers and slightly but not significantly better than telmisartan monotherapy.

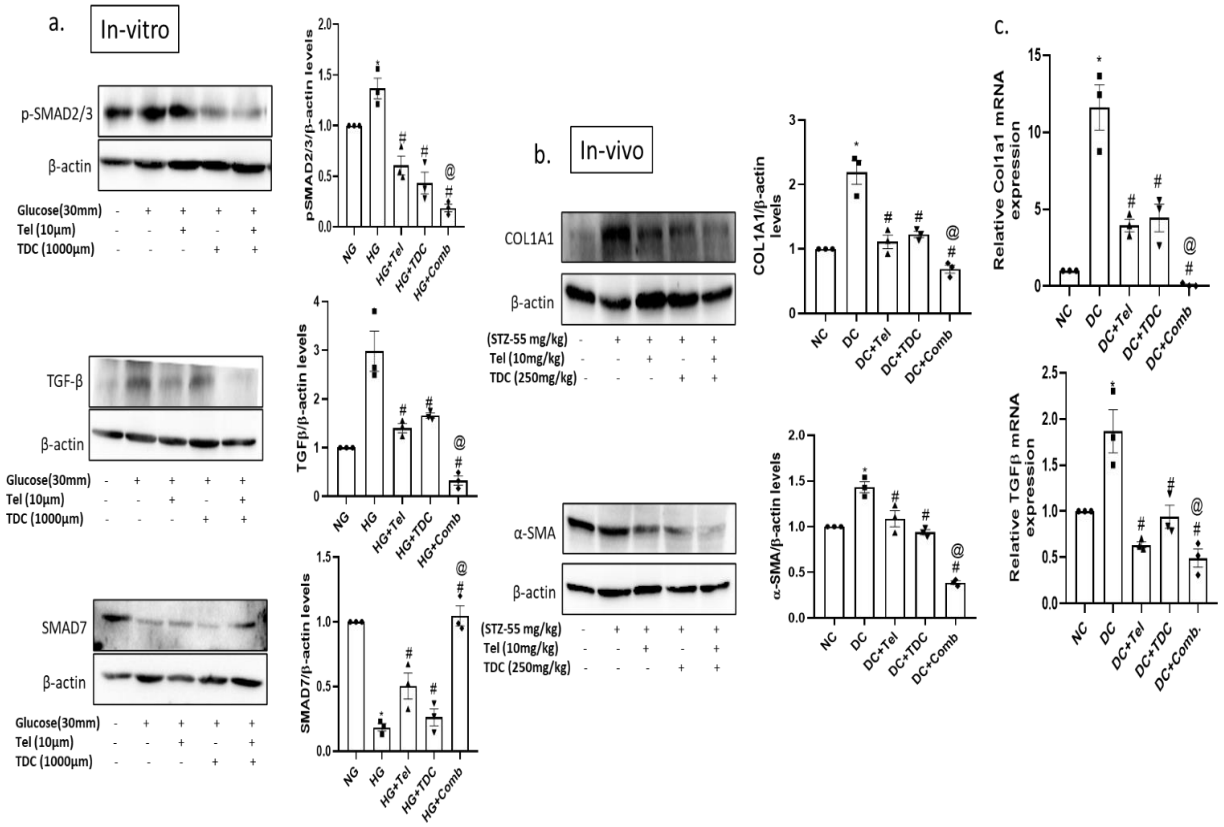


Fig. 25. Effect of the telmisartan and TUDCA monotherapies and combination therapy on profibrotic signaling in the isolated kidney tubules and NRK-52E cells incubated with high glucose. 25a. Representative immunoblot and fold change in the expression of p-SMAD2/3, TGF-β, and SMAD7 in the NRK-52E cells exposed to high glucose and different treatments. **25b.** Representative immunoblot and fold change in the expression of Col1A1 and α-SMA in the kidney tubules. **25c.** Represents the fold change in the expression of the TGF- β and Col1A1 mRNA from the tubules with 18S as the control. All the values are represented as mean ± SEM; n = 3. [(*) $p < 0.05$ vs NC; (#) $p < 0.05$ vs DC; (@) $p < 0.05$ vs monotherapies].

Chapter 6

Discussion

6. Discussion

Diabetes is a chronic disease which is increasing at an alarming rate. As per WHO, diabetes was 9th in the list of leading causes of death with approximately, 1.5 million deaths directly attributed to diabetes in 2019. Diabetes is also threatening since it is a chronic disorder and affects multiple organs over the period of time. We have already discussed the different complications including micro and macrovascular complications associated with diabetes. Despite several years of research and a number of drugs targeting glycemic control, hemodynamic and metabolic pathways the diabetic patients tend to develop different complications. Due to this the researchers are continuously studying the molecular pathways involved in the diabetes and associated complications and novel ways to target the same. ER stress is one such molecular pathway which has emerged as a common link in all the diabetic complications including diabetic endothelial dysfunction, diabetic retinopathy, diabetic neuropathy, DKD etc. Studies are focusing on the factors leading to the development and regulation of ER stress and therapies which can successfully ameliorate the persistent ER stress. We also studied the epigenetic mechanisms involved in the regulation of ER stress associated with hyperglycemia in kidney tubular epithelial cells. 4-PBA, TUDCA, Salubrinal etc. are the few entities used experimentally to reduce the ER tress in different disorders. Out of all these, TUDCA is under clinical trials for diabetes and associated complications including endothelial dysfunction. Besides, the studies pertaining to pathophysiology of diabetes and complications suggest multifactorial etiology which is usually difficult to contain using a single therapy at a time. The combination therapy approach is already in place in the patients with inadequate control with a standard monotherapy, such as combining metformin with any glipizide's, or gliflozins. Furthermore, clinical trials are conducted for triple combination therapies using different classes of antihyperglycemic drugs. Also, use of alternative complimentary medicines *per se* or in combination with conventional medicines for diabetes and associated complications in on all a time high. As per a report approximately 25% to 57% of diabetics report using complementary or alternative medicine (Grossman et al., 2018). TUDCA, being a drug of natural origin and have pleiotropic actions apart from ER stress inhibition. This study evaluated the potential of a combination of TUDCA with RAS modulators, AT1R blockers and ACE2 activators which are well known for their use in diabetic complications but *per se* have not been significantly effective in preventing the diabetic complications. Here we combined TUDCA with diminazene aceturate (ACE2 activator) against diabetic vascular endothelial dysfunction keeping in mind the role of ER stress in the

development of endothelial damage and role of ACE2 and Ang (1-7) in protection of the endothelium. We also evaluated another combination where TUDCA was combined with Telmisartan (AT1R blocker) for the prevention of DKD. In both the studies, we observed that the combinatorial approach proved to be significantly better in preventing the diabetic endothelial dysfunction and DKD respectively as compared to the monotherapies.

6.1. Simultaneous activation of ACE2 and ER stress inhibition significantly reduced the type 1 diabetes induced vascular endothelial dysfunction

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 RAS characterized by elevated angiotensin-II, which is a potent vasoconstrictor and a well-known inducer of oxidative and ER stress. Besides, during hyperglycemia, the protective arm of the RAS consisting of ACE2 and angiotensin (1-7) is suppressed (Senanayake et al., 2018). Previously, angiotensin (1-7) has been found to induce vasodilatation via different mechanisms (Murugan et al., 2015; Zhang et al., 2015). Damage to the vascular endothelium during diabetes is of multiple etiology and hence it is difficult to prevent it using a single therapy.

Here in this study, we have used a combination therapy consisting of an ACE2 activator and ER stress inhibitor to counter the hyperglycemic damage to endothelium in a better way. Streptozotocin-induced T1DM rats showed a significant increase the blood glucose. In monotherapies, TUDCA was found to reduce blood glucose levels. This was in concordance with previous studies where ER stress inhibition using TUDCA has resulted in reduced blood glucose levels (Bronczek, Gabriela Alves et al., 2019; Ozcan et al., 2006). Bronczek et al. attributed the glucose-lowering effect of TUDCA to inhibition of hepatic insulin-degrading enzyme and increasing the activity of this enzyme, thus causing an improvement in the activity of the insulin. Moreover, they also reported that TUDCA has a β cell-protective property, and it increases the β cell number as well as its mass, explaining the anti-hyperglycemic property

of TUDCA (Bronczek, Gabriela Alves et al., 2019). In fact, a recent study showed that persistent ER stress could promote hyperglycemia (Liu et al., 2019). Although, we did not find a significant change in the blood glucose level post ACE2 activation. However, when it was combined with TUDCA, the combination showed additional glucose-lowering ability. Few studies have even reported that ACE2 reduced hyperglycemia owing to properties such as reduced oxidative stress, increased blood flow to the β cells of the pancreas (Chhabra et al., 2013). This could explain the additional anti-hyperglycemic effect of the combination. Impairment in the vasorelaxation is the most important characteristic of endothelial dysfunction.

We also found that prolonged hyperglycemia led to the reduction of Ach-induced vasorelaxation in the aorta. This was in line with previous studies where hyperglycemia has led to reduced vasorelaxation (Romero et al., 2008; Sun et al., 2017). Multiple reasons have been proposed for the same, including elevated oxidative stress and ER stress which leads to the damage of endothelium and reduction in the bioavailability of NO (Imanishi et al., 2006). ER stress inhibition could be beneficial in endothelial dysfunction because they have the capacity to restore the bioavailability of NO, an important factor in maintaining vascular homeostasis. In our study, we found that treatment with TUDCA improved the levels of NO in the aorta of diabetic rats. Studies also suggest that ER stress may induce endothelial cell apoptosis and increase oxidative stress, both responsible for endothelial dysfunction. Also, hyperglycemia may lead to elevated oxidative stress due to increased angiotensin-II and reduced ACE2 and angiotensin (1-7) activity. Such an increase in oxidative stress due to elevated angiotensin-II is also known to reduce NO bioavailability. This was supported by our results where ACE2 activation using diminazene aceturate markedly reduced the oxidative stress levels as observed by improved glutathione and reduced MDA levels. Due to the interlink pathophysiology of ER stress and oxidative stress, we thought of inhibiting both using a combination of drugs. Consequently, we observed that the combination therapy was better than monotherapies in alleviating oxidative stress and elevating the NO bioavailability. Aortic stiffening is also a major contributor to the loss of the vasorelaxant ability of the aorta (Grigorova et al., 2015). Diabetes leads to the deposition of excess collagen in the aorta. In this study, PSR staining showed excessive collagen in the diabetic rat aorta in comparison to that of the normal rat aorta. Moreover, we observed a significant reduction in the collagen deposition in the animals treated with ACE2 activator and ER stress inhibitor. Hyperglycemia-induced elevation in 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 (Fig. 26).

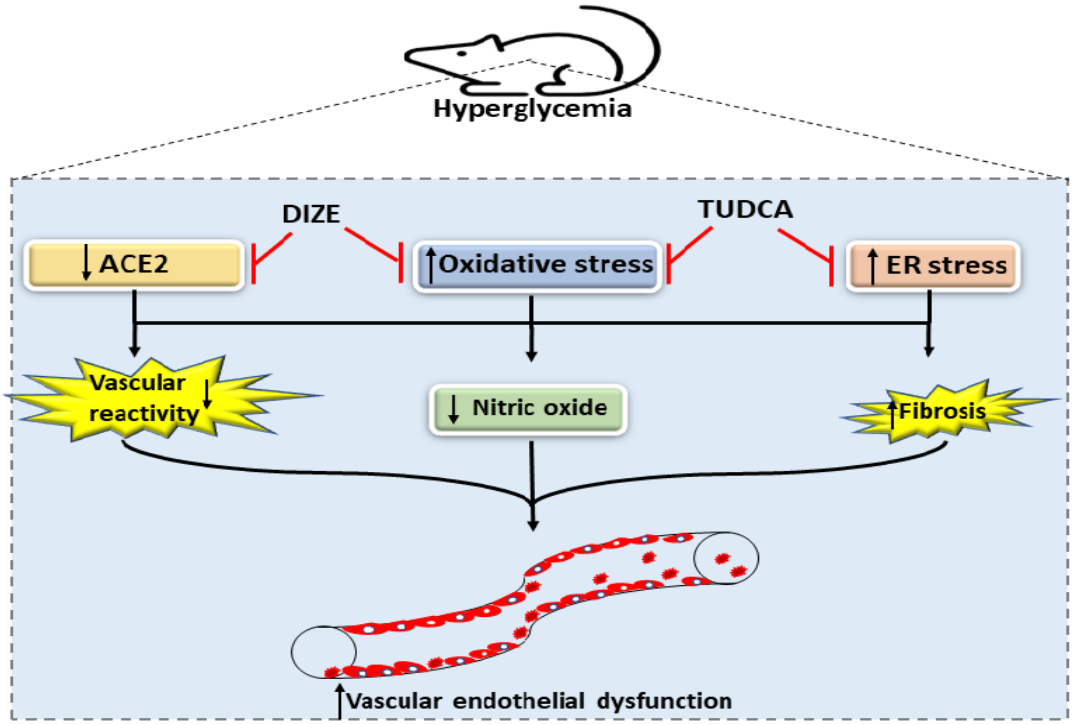


Fig. 26: Schematic representation of the effect of Dize and TUDCA in prevention of vascular endothelial dysfunction.

6.2. Epigenetic regulation of ER stress in the diabetic kidney and high glucose treated NRK-52E cells

Persistent hyperglycemia is a well-known factor in the progression of DKD (Anders et al., 2018; Barrera-Chimal and Jaisser, 2020). Injury to the renal tubular epithelial cells due to high glucose is important to progression of DKD. Also, hyperglycemia is known to modulate the epigenetic machinery such as altered histone methylation pattern such as an increase in the active chromatin marks (H3K4Me1/2/3) or decrease in the repressive chromatin marks (H3K9Me2/3) (Lu et al., 2021). Previously too, change in HMT levels (SET7/9; SUV39H1; Ezh2 etc.) and resulting histone modifications have been linked to renal fibrosis (Lu et al., 2021; Sun, G. et al., 2010). This study corroborates the role of hyperglycemia in epigenetic alteration, where it led to an increase in SET7/9 elevation and consequential increase in active chromatin mark H3K4Me1 and decreased repressive mark H3K9Me2. The study also showed that inhibition of SET7/9 led to amelioration in ER stress, inflammation, and renal fibrosis (Fig. 27). Moreover, we have shown for the first time that Cyproheptadine could be a potential therapeutic entity against DKD. Cyproheptadine, an inhibitor of SET7/9 activity was found to not only reduce the expression of H3K4Me1 but also reduce the ER stress, apoptosis, pro-inflammatory and profibrotic markers in NRK-52E cells thus protecting them.

Cyproheptadine was found to inhibit the SET 7/9 activity in cancer cells (Takemoto et al., 2016). SET7/9 is a known initiator of inflammation and fibrosis, renal and extra-renal (Chen, J. et al., 2014; Sasaki et al., 2016; Tamura and Doi, 2018). Previously, Sasaki et al. found that SET7/9 was associated with the development of TGF-β1 associated renal fibrosis and inhibition of the same using Sinefungin ameliorated the renal fibrosis (Sasaki et al., 2016). Tamura et al. found that SET7/9 has a role in increasing peritoneal fibrosis (Tamura and Doi, 2018). Similar findings regarding the role of SET7/9 in inflammation and ensuing fibrosis were reported by Li et al (Lindenmeyer et al., 2008a). Based on our findings, we hypothesized that Cyproheptadine could be useful against the progression of DKD owing to its SET7/9 inhibitory potential it may protect the NRK-52E cells from hyperglycemia induced injury.

Diabetic rats showed significant deterioration in renal functions as evidenced by a significant elevation in BUN levels, plasma creatinine. Diabetic kidneys showed overexpression of profibrotic (COL4A1, Fibronectin and TGF-β) and pro-inflammatory (p-NF-κB, p-IKKα/β) markers. Moreover, it was found that the expression of SET7/9 is significantly increased in the kidney of diabetic animals. Further, changes in active and repressive chromatin marks from the histone isolated from diabetic kidneys corresponded with the previous findings (Chen, J. et al., 2014; Sasaki et al., 2016). Besides causing epigenetic changes, hyperglycemia also leads to the

generation of ER stress, now recognized as an important mediator in the progression of DKD. ER stress is more pronounced in the tubular portion of the kidney during DKD. Hence, we chose to specifically study the changes in the proximal tubular epithelial cells of the rat kidney (NRK-52E cells).

We observed increased H3K4Me1 in the hyperglycemic NRK-52E cells. Our results are in corroboration of previous findings where hyperglycemia has led to an increase in the level of SET7/9 and resulting histone lysine methylation in different cells. Yunlei D reported that upon exposure to high glucose (25 mM), rat glomerular mesangial cells (HBZY-1) indeed increased the expression of SET7/9. They also observed the resulting increase in the H3K4Me1 (Yunlei et al., 2018). In another study involving vascular endothelial cells, SET7/9 levels were elevated after incubation under high glucose, again followed by higher levels of H3K4Me1 (Okabe et al., 2012). Moreover, we also report that Cyproheptadine significantly suppressed the H3K4Me1 levels in NRK-52E cells. The enhancement in active chromatin marks (K3K4Me1) and decrease in the repressive chromatin mark (H3K9Me2) is responsible for activating the downstream fibrotic and inflammatory signaling cascade.

Further, SET7/9 and H3K4Me1 have been linked to the generation of inflammation. It was found that SET7/9 primarily mediates the hyperglycemia-associated inflammation via activating the transcription factor NF-κB (Paneni et al., 2015). In our study, too, we observed the activation of the said transcription factor as shown by increased expression of p-NF-κB p65. This led to an increase in the pro-inflammatory downstream proteins such as p-IKKα/β. This SET 7/9 mediated inflammatory cascade could potentially activate the renal fibroblast cells leading to fibrosis at the end. As per literature, the presence of inflammatory cells and cytokines is a vital determinant of the mechanism of activation of renal fibroblasts (Kanasaki et al., 2013).

Moreover, the role of hyperglycemia in increasing the activity of TGF-β is well known (Di Paolo et al., 1996; Hayashida and Schnaper, 2004; Li et al., 2003). Precisely, TGF-β signaling could be seen as an important mediator in the progression of renal fibrosis associated with DKD (Chang et al., 2016; Zhao et al., 2020). We also found an increase in TGF-β both *in-vitro* and in the diabetic kidney of rats. Along with that, we also observed reduced SMAD7 (a negative regulator of TGF-β) in the cells incubated with high glucose. Earlier, SET7/9 was reported to activate the TGF-β signaling. When we treated the cells with Cyproheptadine, we observed a marked reduction in the TGF-β and elevation in the levels of SMAD7 indicating the reversal of TGF-β signaling by Cyproheptadine via SET7/9 inhibition.

Furthermore, hyperglycemia and the resulting alteration in TGF-β signaling during DKD also lead to the generation of ER stress (Lee et al., 2015). Post ER stress, UPR is induced to counter the same, and CHOP is an important downstream to the same. Studies report that deletion or reduction of CHOP is in fact, beneficial in DKD and CKD (Carlisle et al., 2021; Wang, 2021). PERK-eIF2α-CHOP signaling has been implicated in the development of diabetes-related fibrosis (Wang, W.W. et al., 2021). In the current study, an increase in the levels of p-eIF2α and CHOP was observed indicating an increase in ER stress. Both hyperglycemia and SET7/9 have been linked to the generation of ER stress individually. Interestingly, in our study ER stress was found to be reduced on Cyproheptadine treatment as evidenced by reduced CHOP and p-eIF2α expression in NRK-52E cells which could be attributed to its SET7/9 inhibitory potential. Moreover, it is well known that insistent hyperglycemia induces cell apoptosis which is vital to the progression of kidney damage to fibrosis. Cyproheptadine successfully reduced the percentage cell apoptosis due to hyperglycemia indicating towards its capability in preventing the progression towards fibrosis (Fig. 27).

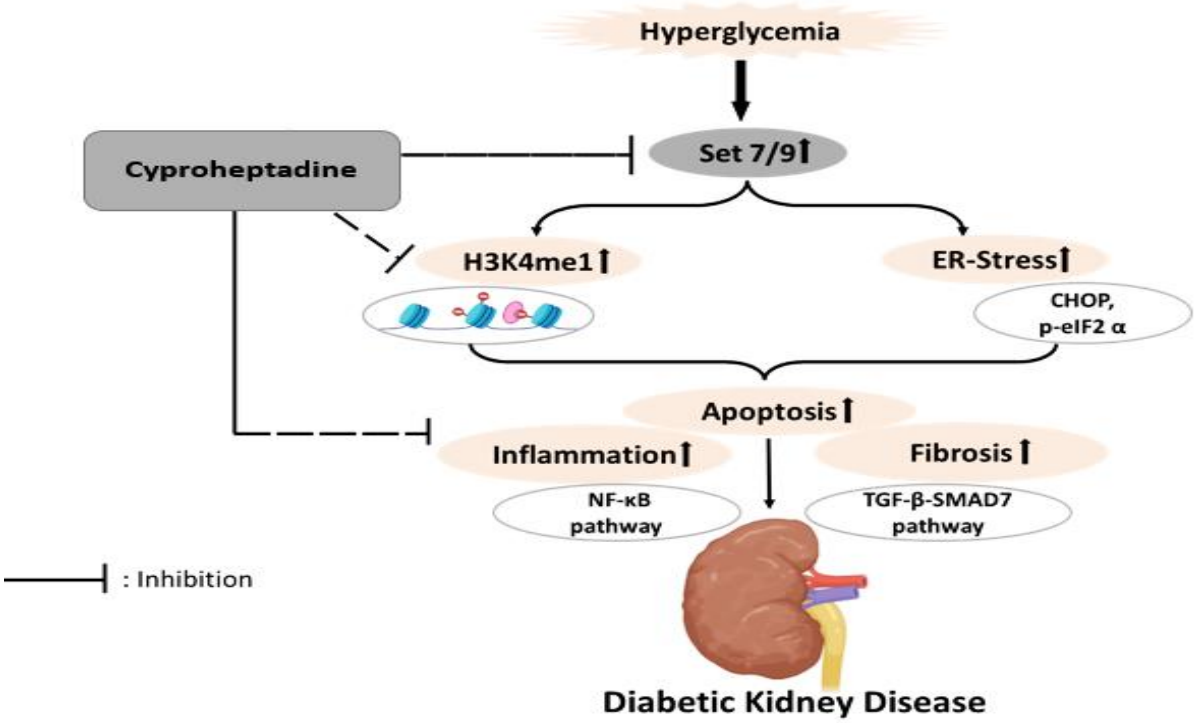


Fig. 27: Effect of hyperglycaemia on expression of SET7/9 and consequent downstream signalling leading to DKD. Hyperglycemia leads to an increase in the activity of SET7/9 which further increases the ER stress, inflammation, apoptosis and fibrosis. Cyproheptadine being a SET7/9 inhibitor could reduce the activity of SET7/9 thereby preventing the downstream signaling and progression towards DKD.

6.3. Effect of the combined treatment of TUDCA and telmisartan on the renal morphology and tubulointerstitial fibrosis associated with DKD

Diabetes is already a global epidemic and consequentially the epidemiology of the complications associated with it is also on the rise. Although there are several medications available for glycaemic control, the number of patients using additional dietary supplements either *per se* or in addition to the existing therapies is high. As per a survey report, 57% of the participating diabetic people agreed to have consumed complementary alternative medicine (Yeung et al., 2018). TUDCA is one of such nutritional supplements which could be taken along with the available therapies to get better outcomes against DKD. Combination therapy is the need of the hour to prevent DKD progression since despite the availability of several therapeutic agents more than half of these patients still progress to ESRD necessitating the search for a novel and effective therapeutic strategy (Ricciardi and Gnudi, 2021; Wang, J. et al., 2021). Several studies have indicated the role of ER stress in mediating the progression of DKD (Cao, A.-L. et al., 2016; Chen et al., 2021; Cunard, 2015). Moreover, the role of RAS overactivation is already established in augmenting the DKD progression including in our previous reports (Goru, S. K. et al., 2017; Malek et al., 2019), and also in the generation of ER stress (Ha et al., 2015; Wang, J. et al., 2015). Therefore, this study was aimed to combine the AT1R blocker and ER stress inhibitor to evaluate any probable additive protection against the DKD progression. The key finding of this study is that a combination of Telmisartan and TUDCA significantly ameliorated the DKD and prevented the decline in renal function when compared to the respective monotherapies.

Chronic persistent hyperglycemia which is vital to the progression of DKD is also a known inducer of ER stress (Lindenmeyer et al., 2008b). We used streptozotocin to develop DKD. We observed a steep decline in renal function such as high blood urea nitrogen, and high plasma creatinine in the diabetic animals at the end of 8 weeks. Also, the kidney weight to body weight ratio of the animals was high indicating kidney hypertrophy. The observed biochemical and morphometric changes were in line with our previous studies (Goru, S. K. et al., 2017; Malek et al., 2019).

On treatment with telmisartan and TUDCA, besides reduction in the BUN and creatinine, both the monotherapies and the combination therapy significantly reduced the blood glucose level. This could be explained since TUDCA is previously reported to reduce blood glucose levels by preventing the loss of the β cell mass and reducing the degradation of insulin-degrading

enzymes in the liver (Bronczek, G. A. et al., 2019). Telmisartan is already known to reduce blood glucose by enhancing insulin secretion via different mechanisms (Liu et al., 2021). The different blood-glucose-lowering mechanisms of the two drugs explain the additive lowering of blood glucose when administered in combination. The animals in the diabetic group also showed deterioration in the morphology of the kidney including damaged and necrotic tubules as can be seen in the haematoxylin and eosin images. Furthermore, the kidney tubules also showed deposition of collagen in the sirius red stain which was higher in diabetic animals but significantly reduced in the treated groups. This is due to the metabolic changes associated with the diabetes which disrupts the renal haemodynamic and promotes mesangial expansion, inflammation, tubulointerstitial fibrosis etc (Alicic, R. Z. et al., 2017). Both TUDCA and telmisartan have the ability to prevent the disruption in the normal physiology by providing glycaemic control and regulating the RAS components. Besides, hyperglycaemia associated with the DKD and dysregulation of RAS is known to cause ER stress.

Previously, an increase in ER stress has been observed in diabetic animals as well as the cells exposed to the hyperglycaemic environment. The reason for an increase in the ER stress in diabetic animal's ranges from hyperglycemia, and proteinuria to increased advanced glycation end product and free fatty acid formation (Allen et al., 2003; Chen, Y.-W. et al., 2011). Also, the dysregulation of RAS in such animals leads to increased levels of angiotensin II which itself is a prominent ER stress inducer (Ha et al., 2015). In line with these reports, we also observed an increase in ER stress as indicated by the increase in the expression of UPR mediators such as BiP, p-eif2α, CHOP, etc. both in the kidney tubules from the rats as well as the NRK-52E cells. Telmisartan and TUDCA both significantly reduced the ER stress. Interestingly, the combination of the two brought down the ER stress further indicating an additive effect. This could be because, besides the inhibition of angiotensin-induced ER stress by telmisartan and TUDCA, TUDCA can increase the angiotensin-(1-7) which might provide additional protection against ER stress.

We also observed a marked increase in RTN1a in the tubular cells of the rat kidney. RTN1a has recently been shown as a prominent inducer of ER stress and vital to the kidney damage associated with DKD (Fan et al., 2017). Interestingly, both telmisartan and TUDCA significantly ameliorated the expression of RTN1a which was more prominent in the combined therapy of these two drugs. This could potentially be a reason that the combination of the drugs showed an additive effect in alleviating apoptosis and fibrosis associated with DKD.

Chronic hyperglycemia has been reported to cause apoptosis both *in-vivo* and *in-vitro* due to different mechanisms such as increased oxidative stress (Allen et al., 2003), activation of NF-κB and p53 pathways (Chen, Y.-W. et al., 2011), etc. Unresolved and persistent ER stress leads to the activation of the transcription factor CHOP which ultimately leads to the apoptosis of kidney cells (Shahzad et al., 2021). In the current study also, it was observed that mRNA and protein expression of the apoptotic markers such as cleaved-PARP, caspases, etc was significantly enhanced. Further, both the monotherapies TUDCA and telmisartan reduced the apoptosis significantly in comparison to the diseased group. Also, the combined therapy prevented apoptosis significantly better than the monotherapies.

Kidney fibrosis is the hallmark of chronic kidney diseases including DKD (Border and Noble, 1998; Chen et al., 2021). There is increasing evidence that suggests that activation of ER stress and consequent UPR leads to the development of fibrosis (Chiang et al., 2011). We also observed an increase in the mRNA and protein expression of TGF-β and reduced SMAD7 which is a negative regulator of TGF-β. TGF- β signaling is critical to the development of renal fibrosis. Moreover, interplay has been found to occur between the TGF- β signaling and ER stress in the kidney as well as in other diseases (Ke et al., 2017). On one hand TGF- β has been reported to cause ER stress and on the other persistent ER stress was found to activate the TGF-β signaling. Also, studies report that regulation of TGF- β via RAS signaling could also prove useful in kidney fibrosis (Border and Noble, 1998). Hence, targeting both RAS and ER stress simultaneously to effectively control the progression of kidney fibrosis was the aim and we obtained favorable results wherein there was a marked reduction in deposition of collagen, and expression of TGF- β, COL1A1, pSMAD2 more effectively by the combination therapy as compared to the monotherapies. Overall, in the majority of the parameters evaluated TUDCA alone brought a significant improvement and the combination therapy further improved the disease outcome (Fig. 28).

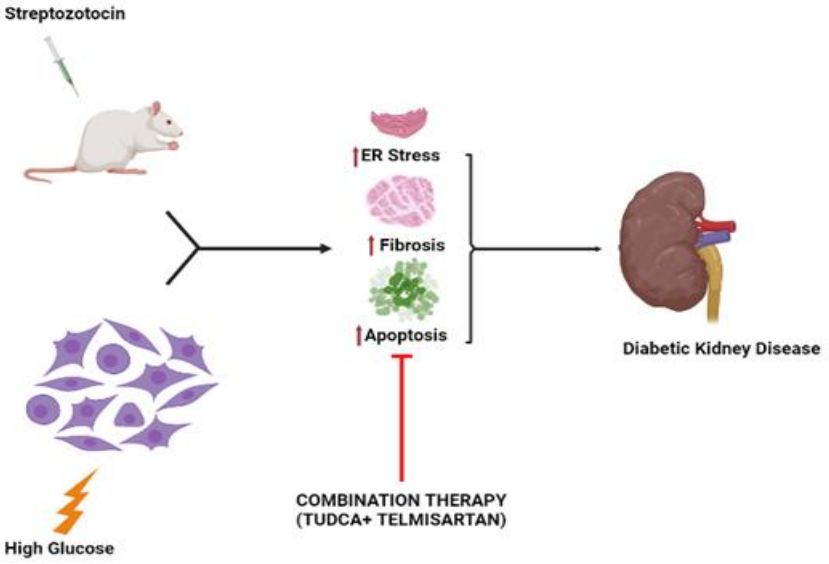


Fig. 28: Combination therapy of telmisartan and TUDCA provides better protection to the kidney as compared to monotherapy.

Overall, in the current study we explored the role of ER stress and the mechanism via which it is regulated. We observed that apart from usual environmental and pathological cues epigenetic machinery is also involved in the regulation of ER stress. We explored the role of SET7/9 in particular in regulating the hyperglycaemia associated ER stress and found that targeting SET7/9 using specific inhibitors could prove useful in countering the DKD progression. Moreover, we also combining a nutraceutical in the form of TUDCA, with an AT1R blocker and ACE2 activator proved to be a more effective way in countering the diabetic complications, endothelial dysfunction and DKDs in the current study. TUDCA leads to the reduction of ER stress, apoptosis, fibrosis associated with persistent hyperglycaemia. The dysregulation of RAS is known to elevate the ER stress. Several studies have reported to an increase in ER stress owing to increased angiotensin-II in kidney as well as endothelium. So, combining an ER stress inhibitor with RAS modulators seemed to be a rational approach in preventing the progression of diabetic complications. Results obtained in this study also corroborate the usage of the TUDCA in combination with RAS modulators. The limitations and future prospective of the current study have been discussed separately.

Chapter 7

Conclusion

7. Conclusions

- Vascular endothelial dysfunction due to chronic hyperglycemia during diabetes is a major risk factor for development of diabetic CVD. We concluded 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. 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.
- Our results concur that hyperglycemia may add on to the progression of DKD by injuring NRK-52E cells via an increase in SET7/9 and ER stress. Moreover, we provided a base for further studies regarding the use of Cyproheptadine, a clinical entity in diabetic conditions to prevent the deterioration of kidney health. We also tested the hypothesis that Cyproheptadine might prevent the damage to NRK-52E cells owing to its SET7/9 inhibitory potential. We revealed that Cyproheptadine successfully attenuated the hyperglycemia-associated damage to tubular epithelial cells by reducing H3K4Me1 and ameliorating ER stress and associated inflammation, apoptosis and fibrosis. SET7/9 could prove to be a crucial target for the development of a therapeutic strategy against DKD. Cyproheptadine or the moieties based on it could be developed as a novel SET7/9 inhibitor for clinical use.
- TUDCA supplementation significantly potentiated the renoprotective activity of telmisartan. Diseases such as DKD, which has complex etiology, are relatively difficult to control using a single therapy. Hence, combining different forms of treatments, including dietary supplements with strict hyperglycemic control, RAS inhibitors, SGLT2 inhibitors, etc., is considered to be the newer approach to controlling the progression of DKD efficiently. In the same vein, we thought of combining the AT1R blocker with ER stress inhibitor to apply a multipronged approach to countering DKD and found that it significantly reduced the DKD progression compared to the respective monotherapies. Since ER stress and RAS signaling are interlinked, ER stress plays a significant role in DKD pathophysiology. Also, TUDCA is safe, well-tolerated, and can be combined with telmisartan, one of the widely prescribed drugs in DKD patients. Therefore, it could prove to be a novel therapeutic strategy against DKD.

Chapter 8

Limitations and Future Prospective

8. Limitations and Future Prospective

- We showed that a combination therapy of ACE2 activation using Dize and ER stress inhibition using TUDCA was significantly better in preventing the endothelial dysfunction as compared to the monotherapies. Although, we showed the therapeutic relevance of the combination therapy but the underlying molecular mechanisms still need to explored further. In future, apart from the overall protective effect of TUDCA on endothelium, its effect on the different RAS components such as ACE2, Ang (1-7) needs to be evaluated. It could be possible that the combination therapy produced a better effect due to TUDCA also activating the protective arm and suppressing the pressor arm of RAS besides ER stress inhibition. Further, the vice-versa experiments should also be performed to evaluate the effect of Dize on ER stress inhibition via ACE2 activation.

- We explored the epigenetic mechanism via which ER stress is regulated in kidney cells under hyperglycemic conditions. We found that hyperglycemia leads to the increased expression of SET7/9, a histone methyl transferase which further leads to an increase in ER stress. However, we did not evaluate the effect of the hyperglycemia on the enzymatic activity of the SET7/9. Further we also showed that Cyproheptadine, could be used as a SET7/9 inhibitor in the prevention of hyperglycemia induced ER stress in NRK-52E cells, but we could not explore the same in animal model. In future studies the effect of cyproheptadine should be evaluated in the suitable animal models for its SET7/9 inhibitory potential. Further preclinical and clinical exploratory studies may help in repurposing cyproheptadine as well as developing new molecules targeting SET7/9 for their use in DKD and other fibrotic disorders.

- We evaluated the potential of a combination of an ER stress inhibitor (TUDCA) with AT1R blocker (Telmisartan) in the prevention of DKD. We observed better renoprotection by the combination therapy than the respective monotherapies both *in-vitro* and *in-vivo.* Further studies need to be conducted regarding the dose ratio of the two drugs, pharmacokinetics of the drugs and their interaction before moving on to the clinical trials. TUDCA is safe and well tolerated available over the counter too as supplements, so it has the potential to be taken with conventional therapeutics.

Chapter 9

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9. References

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Appendix

Appendix I

List of Publications from Thesis

1. **Sankrityayan H**, Kale A, Gaikwad AB, 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. Canadian Journal of Physiology and Pharmacology. (2022) 100: 234–239 [IF- 2.245]
2. **Sankrityayan H**, Kale A, Shelke V, Gaikwad AB, Cyproheptadine, a SET7/9 inhibitor, reduces hyperglycemia-induced ER stress alleviating inflammation and fibrosis in renal tubular epithelial cells. Archives of Physiology and Biochemistry (2022) [IF- 3.188]
3. **Sankrityayan H**, Shelke V, Kale A, Gaikwad AB, Evaluating the potential of tauroursodeoxycholic acid as add-on therapy in amelioration of streptozotocin-induced diabetic kidney disease. (Revision requested, European Journal of Pharmacology)
4. **Sankrityayan H**, Oja MJ, Kulkarni YA, Mulay SR, and Gaikwad AB, ER stress response mediates diabetic microvascular complications, Drug Discovery Today, 24 (12) (2019) 1-15. [Impact Factor: 8.369]
5. **Sankrityayan H**, Gaikwad AB, Diabetic nephropathy: The regulatory interplay between epigenetics and microRNAs. Pharmacological Research, 141 (2019) 574–585 [IF- 10.334]
6. **Sankrityayan H**, Rao PD, Shelke V, Kulkarni YA, Mulay SR, Gaikwad AB. Endoplasmic Reticulum Stress and Renin-Angiotensin System Crosstalk in Endothelial Dysfunction. Current Molecular Pharmacology, 2022. [Impact Factor: 3.855]

List of Other Publications

1. Malek V, Sharma N, **Sankrityayan H**, and Gaikwad AB, Concurrent neprilysin inhibition and renin-angiotensin system modulations prevented diabetic nephropathy. Life sciences 221 (2019) 159-167. [Impact Factor: 6.780]
2. Sharma N, **Sankrityayan H**, Kale A, and Gaikwad AB, Role of SET7/9 in the progression of ischemic renal injury in diabetic and non-diabetic rats. Biochemical and Biophysical Research Communications 528, 1 (2020) 14-20. [Impact Factor: 3.322]
3. Rao PD, **Sankrityayan H**, Srivastava A, Kulkarni YA, Mulay SR, and Gaikwad AB, ‘PARP’ing fibrosis: repurposing poly (ADP ribose) polymerase (PARP) inhibitors. Drug discovery today (2020) 1253-1261. [Impact Factor: 8.369]

4. Kale A, **Sankrityayan H**, Anders HJ, and Gaikwad AB, Epigenetic and non-epigenetic regulation of Klotho in kidney disease. Life Sciences 264 (2021) 118644. [Impact Factor: 6.780]
5. Kale A, **Sankrityayan H**, Anders HJ, and Gaikwad AB, Klotho in kidney diseases: A crosstalk between the renin-angiotensin system and endoplasmic reticulum stress. Nephrology Dialysis Transplantation (2021). [Impact Factor: 7.186]
6. Kale A, **Sankrityayan H**, Anders HJ, and Gaikwad AB, Klotho: A possible mechanism of action of SGLT2 inhibitors preventing episodes of acute kidney injury and cardiorenal complications of diabetes. Drug Discovery Today (2021). [Impact Factor: 8.369]
7. Kale A, **Sankrityayan H**, and Gaikwad AB, Epigenetic restoration of endogenous Klotho expression alleviates acute kidney injury-diabetes comorbidity. Life Sciences 288 (2022) 120194. [Impact Factor: 6.780]
8. Shelke V, Kale A, **Sankrityayan H**, Anders HJ and Gaikwad AB, Long non-coding RNAs as emerging regulators of miRNAs and epigenetics in diabetes-related chronic kidney disease. Archives of Physiology and Biochemistry. https://doi.org/10.1080/13813455.2021.2023580. [Impact Factor: 3.188]
9. Kale A, Shelke, V, **Sankrityayan H**, Dagar, N, and Gaikwad AB, Klotho restoration via ACE2 activation: A potential therapeutic strategy against acute kidney injury-diabetes comorbidity. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, (2022) 166532. [Impact Factor: 6.63]

List of Conferences other than thesis

1. **Sankrityayan H**, Malek V, Sharma N, "Simultaneous Inhibition of Neprilysin and Activation of ACE2 prevented diabetic cardiomyopathy". **International Conference of Cardiovascular Sciences-2020** (ICCS-2020), February 2020, DPSRU-New Delhi, India. (Oral presentation- **won CC Kartha travel Grant Award**).

Appendix II: Biographies

Brief Biography of the Supervisor

Prof. Gaikwad Anil Bhanudas is an Associate Professor of Pharmacy at BITS Pilani, Pilani campus. He is also working as Associate Dean of Practice School Division. He did his Masters and Ph.D. from the Department of Pharmacology and Toxicology, NIPER, SAS Nagar. He was awarded Doctoral Sandwich Fellowship from DAAD (German Academic Exchange Services) during his doctoral studies. He visited reputed overseas institutes as visiting scientist in the Department of Medicine/Nephrology, Albert Einstein College of Medicine, NY, USA, and Nephrological Center, Medizinische Poliklinik, Ludwig-Maximilians-University, Munich, Germany, in 2010 and 2008, respectively. His research grants are from SERB, UGC, DBT, ICMR, and CSIR. To date, he has provided essential and novel evidence on histone post-translational modifications and the protective axis renin-angiotensin system in the development of diabetic kidney diseases. He has contributed to several book chapters published by Elsevier and has 60 peer-reviewed research publications such as Drug Discovery Today (2021, IF: 7.85) Cardiovascular Research (2020, IF: 10.78), British Journal of Pharmacology (2017, IF: 8.74). He has supervised one PDF and five Ph.D. students and, at present, is guiding four Ph.D. students. He is also serving as an Associate Editor for Frontiers in Endocrinology Journal (Cardiovascular Endocrinology section) and BMC Pharmacology and Toxicology Journal. Further, he is also a Review Editor for the Renal Pharmacology section of the Frontiers in Pharmacology Journal.

Brief Biography of the Candidate

Mr. Himanshu Sankrityayan has graduated in Pharmacy from Bundelkhand University, Jhansi, Uttar Pradesh, India in the year 2012. He completed his M. Pharm. degree in Pharmacology and Toxicology from the Bombay College of Pharmacy, Mumbai, Maharashtra, India in the year 2015. He has joined Laboratory of Molecular Pharmacology, Department of Pharmacy, Birla Institute of Technology and Science Pilani (BITS Pilani), Pilani Campus to pursue his doctoral research work. His areas of interest include chronic kidney disease including diabetic kidney diseases, endoplasmic reticulum stress in diabetic complications, acute kidney injury, and epigenetics. Moreover, he has published 5 original research and 10 review articles in various reputed, international, peer-reviewed journals. He has received a travel grant to attend national conferences.

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Cyproheptadine, a SET7/9 inhibitor, reduces hyperglycaemia-induced ER stress alleviating inflammation and fibrosis in renal tubular epithelial cells

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ORIGINAL ARTICLE

Cyproheptadine, a SET7/9 inhibitor, reduces hyperglycaemia-induced ER stress alleviating inflammation and fibrosis in renal tubular epithelial cells

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ABSTRACT

Context: Persistent hyperglycaemia increases SET7/9 expression and endoplasmic reticulum (ER) stress which causes inflammation, apoptosis, and fibrosis in renal tubular epithelial cells leading to diabetic kidney disease (DKD).

Objective: Current study explores the renoprotective potential of a novel SET7/9 inhibitor, Cyproheptadine, and the underlying molecular mechanisms in hyperglycaemia-induced renal tubular epithelial cell injury.

Methods: Change in expression of SET7/9, histone H3 lysine (K4) monomethylation (H3K4Me1), inflammatory, fibrotic, and ER stress proteins were evaluated *in-vivo* and *in-vitro*. NRK-52E cells were used to study the preventive effect of Cyproheptadine against hyperglycaemia-induced ER stress and subsequent inflammation and fibrosis.

Results: SET7/9 and H3K4Me1 expression significantly increased with ER stress, inflammation, apoptosis, and fibrosis, *in-vivo* and *in-vitro* under hyperglycaemia. However, the cells treated with Cyproheptadine showed significant suppression of H3K4Me1 and reduction in ER stress, inflammation, apoptosis, and fibrosis.

Conclusion: Cyproheptadine prevented hyperglycaemia-induced renal fibrosis and inflammation by reducing H3K4Me1 expression and ER stress.

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Cyproheptadine; SET7/9; ER stress; histone H3 lysine K4 mono-methylation; diabetic kidney disease

Introduction

Diabetic kidney disease (DKD) is a chronic kidney disorder and is primarily responsible for the development of end-stage renal disease (ESRD) (Fu *et al.* 2019). The cases of DKD are on a constant uprise which could be attributed to the epidemiological proportion increase in diabetic patients across the globe (Alicic *et al.* 2017). Several factors are responsible for developing DKD, hyperglycaemia, and hypertension being the most prominent ones (Alicic *et al.* 2017, Lin *et al.* 2018). Downstream the persistent hyperglycaemia lies the inflammatory pathways and the consequential activation of the profibrotic pathways leading to renal fibrosis and culminating in ESRD (Kanasaki *et al.* 2013, Wada and Makino 2013, Furuya *et al.* 2019). Apart from strict glycaemic control, renin-angiotensin system (RAS) inhibitors have been used in preventing the progression of DKD for decades, albeit with much success (Yamazaki *et al.* 2021). The primary characteristics of DKD include hypertrophy in the glomerulus, tubulointerstitial fibrosis, mesangial expansion, etc. Moreover, injury to the tubular epithelial cells is vital to the progression of DKD. Hyperglycaemic exposure acts as an initiator of tubular cell injury (Tong *et al.* 2018). Tubular epithelial cells are prone to the development of ER stress, which is produced due to persistent hyperglycaemia (Fang *et al.* 2013), thus aggravating DKD.

DKD is a disease of multiple aetiologies, and in the past few decades, the role of epigenetics in regulating critical genes involved in the DKD has come to the fore (Lu *et al.* 2021). Epigenetics consists of a change in the expression and function of the gene without alteration in the genetic sequence and is also heritable. For instance, post-translational histone modifications (PTHMs) such as acetylation, methylation, phosphorylation, and ubiquitylation are essential in regulating chromatin structure, and hence genomic DNA accessibility (Kato and Natarajan 2019). A set of different enzymes carries out these modifications, HMTs (Histone Methyl Transferases) for histone methylation, Histone Acetyl Transferases (HATs) and Histone Deacetylases (HDACs) etc. (Kato and Natarajan 2019, Zheng *et al.* 2021). SET domain-containing lysine methyltransferase 7/9 (SET7/9) is one such enzyme that has been implicated in the development of fibrosis lately (Sasaki *et al.* 2016, Tamura *et al.* 2018). SET7/9 is a histone methyltransferase that primarily induces histone H3 lysine (K4) monomethylation (H3K4Me1). Studies showed that transforming growth factor-beta 1 (TGF-β1) is involved in the aggravated expression of SET7/9, and the consequent increase in H3K4Me1 may add to the fibrotic cascade (Guo *et al.* 2016, Sasaki *et al.* 2016, Shima *et al.* 2017).

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Hyperglycaemia has also been implicated in the generation of ER stress which adds to the progression of DKD (Lindenmeyer *et al.* 2008, Wang *et al.* 2021). ER stress also regulates the inflammatory cascade in type 1 diabetic conditions (Qi *et al.* 2011). Moreover, SET7/9 has been reported to regulate the ER stress in hepatocytes, and vice-versa has been found in DKD (Chen *et al.* 2014, Han *et al.* 2021). Overall, there exists an intricate relationship between SET7/9 expression, ER stress and hyperglycaemia, which further regulates the downstream inflammatory and fibrotic pathways. Recently, Cyproheptadine, a clinically approved drug, has emerged as a SET7/9 inhibitor in a few studies (Takemoto *et al.* 2016, Hirano *et al.* 2018). Takemoto *et al.*, in their study, used a high throughput HMT assay based on fluorogenic substrates to screen for the inhibitors of SET7/9. Interestingly, amongst several compounds screened by them, they found Cyproheptadine to be an outstanding candidate for the inhibition. During confirmatory studies, it was found that Cyproheptadine inhibits SET7/9 activity with a half-maximum inhibitory concentration (IC50) value of 1.0 μM (Takemoto *et al.* 2016).

Increased expression of SET7/9 and ER stress in renal tubular epithelial cells has proved to be a significant determinant in the development of renal fibrosis associated with DKD. Therefore, we hypothesised to evaluate the potential of Cyproheptadine in preventing hyperglycaemia-induced damage to the renal tubular epithelial cells and the underlying molecular mechanisms.

Material and methods

Materials

Biochemical estimation kits for Glucose, Blood Urea Nitrogen (BUN), Creatinine were purchased from Accurex (Mumbai, India). Streptozotocin (STZ) was purchased from Sigma-Aldrich (St. Louis, Missouri, United States), and Cyproheptadine was procured from Tocris Biosciences (Bristol, United Kingdom). The rest of the chemicals were purchased from Sigma unless otherwise specified.

Development of renal fibrosis in type 1 diabetic animals

The Institutional Animal Ethics Committee approved all animal care and experimental procedures, Birla Institute of Technology and Science, Pilani (BITS Pilani) [IAEC/RES/25/14/Rev-1/27/15]. Animal studies are reported in compliance with the ARRIVE-2 guidelines. Male adult Wistar rats (180–220 g) were supplied by the central animal facility of BITS Pilani. Animals were maintained under standard environmental conditions and provided with food and water *ad libitum*. Post acclimatisation, animals were administered with a single dose of STZ [55 mg/kg, *i.p.*] for inducing type 1 diabetes. All the experiments were performed at the Central Animal Facility (CAF), BITS Pilani, Pilani Campus, Rajsthan. Sodium citrate buffer (pH-4.4) was injected into the control animals. At the end of 8 weeks post-induction, the animals were sacrificed using an overdose of anaesthesia (pentobarbital sodium), followed by plasma collection and kidneys were removed for further processing.

Biochemical, metabolic, morphometric, and urinary parameters evaluation

The biochemical and urinary parameters, including plasma glucose, plasma creatinine, serum creatinine, urine creatinine, and BUN were estimated as a marker of renal dysfunction using commercially available Accurex kits. Furthermore, daily water consumption, total urine volume, and creatinine clearance were also evaluated as described previously (Agil *et al.* 2020). Change in the animals' body weight with time and kidney weight: body weight ratio was also determined to establish the development of type 1 diabetes and renal fibrosis.

MTT (3–(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide) assay

MTT assay was performed using Cyproheptadine in different doses as previously described (Sharma *et al.* 2019). Briefly, 3000 cells were seeded in 96 well plates and allowed to adhere and grow. Post incubation, the cells were serum-starved for 12 h. After 12 h, cells were incubated with different concentrations of Cyproheptadine ranging from 0.1 μM-20 μM for 48 h. The drugs were aspirated, and the cells were treated with 1 mg/ml of MTT for 4 h. The formazan crystals were solubilised in DMSO, and the absorbance was taken at 570/630 nm.

Cell culture studies

Rat proximal renal tubular epithelial cells (NRK-52E) were obtained from National Centre for Cell Science, Pune. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (containing five mM D-glucose; HiMedia, Carlsbad, CA, USA) containing 10% foetal bovine serum (Invitrogen) at 37 °C with 5% CO_2. The control (normal glucose, NG) group was the serum-free medium of normal glucose DMEM with 5 mM D-glucose, and the HG group was the serum-free medium of HG-DMEM with 30 mM D-glucose. The HG medium was used to mimic the model of type 1 diabetes *in-vitro*. The cells were seeded in 6 well plates and, after serum starvation of 12 h. was exposed to NG, HG and HG with Cyproheptadine (1 μM) for 48 h. Post 48 h., the cell lysate was collected for further processing.

Protein isolation (cytoplasmic and histone) and immunoblot analysis

Rat whole kidney homogenate or cells grown in six-well dishes were lysed in RIPA buffer and sonicated for 3 cycles of 10 s. Histones were isolated from the whole kidneys using the acid extraction method, and Immunoblotting was performed as previously described (Chen *et al.* 2011). Briefly, primary antibodies used in this study were anti-SET7/9 (Cell Signalling Technology, Danvers, MA), TGF-β (Cell Signalling

Technology), p-IKKα/β and p- IκBα (Cell Signalling Technology), p-NF-κB and NF-κB (Cell Signalling Technology), anti-H3K4Me1 (Cell Signalling Technology), anti-histone H3 (Cell Signalling Technology), SMAD7, Col4A1, Fibronectin and anti-β-actin (Santa Cruz Biotechnology). Secondary antibodies used in this study were horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (Santa Cruz Biotechnology) or goat anti-mouse immunoglobulin G antibody (Santa Cruz Biotechnology). All the primary antibodies were used in a dilution of 1:1000 (v/v) whereas the dilution used for secondary antibodies was (1:10000). Signals were detected using the ECL reagent (Bio-Rad). The intensity of each band was determined using ImageJ software (version 1.46r; National Institutes of Health, Bethesda, MD).

Immunofluorescence assay

For immunofluorescence assay, NRK-52E cells were seeded on coverslips. Post incubation the cells were exposed to normal glucose (5 mM), high glucose (30 mM), and high glucose with Cyproheptadine (1 μM) for a period of 48 h. Later, the cells were fixed with 2% paraformaldehyde for 15 min and permeabilized using 0.2% Triton X-100. Further, the cells were blocked with 3% BSA in PBS for an hour and incubated with anti-CHOP primary antibody (1:2000 dilution, CST) overnight at 4° C. Next day; the coverslips were washed with ice-cold PBS thrice for 5 min each and incubated with Alexa-Fluor488 anti-mouse secondary antibody (1:2000 dilution, CST) for 90 min at room temperature. Coverslips were washed thrice from PBS and finally incubated with DAPI for 10 min at room temperature. Finally, the coverslips were mounted at the glass slides and visualised using a Zeiss Confocal laser scanning microscope (Li *et al.* 2017).

RNA isolation and real-time polymerase chain reaction (RT-PCR)

RNA isolation and RT-PCR were performed as described previously (Chen *et al.* 2011). Briefly, RNA was isolated from the whole kidney homogenate using the Trizol method followed by cDNA synthesis using GeneSure H- Minus First Strand cDNA Synthesis Kit. Further, the cDNA was reverse transcribed, and RT-PCR was performed using the iTaq Universal SYBR Green Supermix (Bio-Rad, USA). Relative expression of each gene of interest was evaluated on LightCycler® 96 Real-Time PCR System (Roche) using LightCycler Software (Roche). The abundance of targeted mRNA was normalised against GAPDH or 18 s mRNA.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (8.0.2). To evaluate the significant difference between groups, a t-test was used when two groups were compared and a one-way analysis of variance (ANOVA) while comparing more than two groups. Statistical significance was considered when $p < .05$. All the results were expressed as mean ± SEM.

Results

Cyproheptadine dose determined using cell viability assay

Cell viability assay using MTT was used to determine the cytotoxic potential. It helps in deciding the dose of the drug to be used in the study. In this assay, Cyproheptadine was used starting from the initial dose of 0.1 μM up to 20 μM. Cell viability of more than 75% was observed in 0.1, 0.5 and 1 μM Cyproheptadine (Figure S1). Thus, we chose the dose of 1 μM for our study, since 1 μM was reported to be the IC50 value of the Cyproheptadine against SET7/9.

Morphometric, metabolic and biochemical changes in the animals with DKD

Post 48 h. of STZ administration, all the animals developed hyperglycaemia with plasma glucose levels <21 mmol/l. Markers of the kidney damage including BUN, plasma and serum creatinine were also on the higher side in the type 1 diabetic animals compared to the normal control (Table 1). Type 1 diabetic animals showed a significant increase in water consumption, urine output, urinary creatinine and decreased creatinine clearance (Table 2) as compared to the normal control animals. In type 1 diabetic animals, there was a drastic reduction in body weight after 8 weeks of diabetes. Also, the kidney weight of the type 1 diabetic animals was significantly higher along with the kidney weight and body weight ratio (Table 1). All the above parameters indicate the presence of progressive kidney damage.

Increase in the expression of SET7/9 and H3K4Me1 and decrease in H3K9Me2 in the diabetic kidney and NRK-52E cells under hyperglycaemic condition

Hyperglycaemia is known to cause epigenetic changes in the kidney. Several HMTs such as Enhancer of zeste homolog 2 (Ezh2), Suppressor of Variegation 3–9 Homolog 1 (SUV39H1), G9a, SET7/9 etc. are reported to regulate the progression of kidney diseases (Yu and Zhuang 2019). We found a significant increase in the level of SET7/9 in the kidney of type 1 diabetic animals and the NRK-52E cells incubated with high glucose (30 mM) for 48 h. This indicates the role of hyperglycaemia in increasing the histone methylation marks (H3K4Me1) and adding to the progression of DKD. We observed higher expression of H3K4Me1 in the hyperglycaemic NRK-52E cells as well as diabetic kidney compared to the respective normal controls which is the active chromatin mark and activates the genes responsible for the progression of DKD (Figure 1(a,b)). A decrease in H3K9Me2 was also found in the histones isolated from kidneys of type 1 diabetic rats indicating a decrease in repressive genes again promoting DKD advancement (Figure 1(c)). This increase in the active chromatin marks and decrease in the repressive chromatin marks leads to the activation of the fibrotic and inflammatory genes.

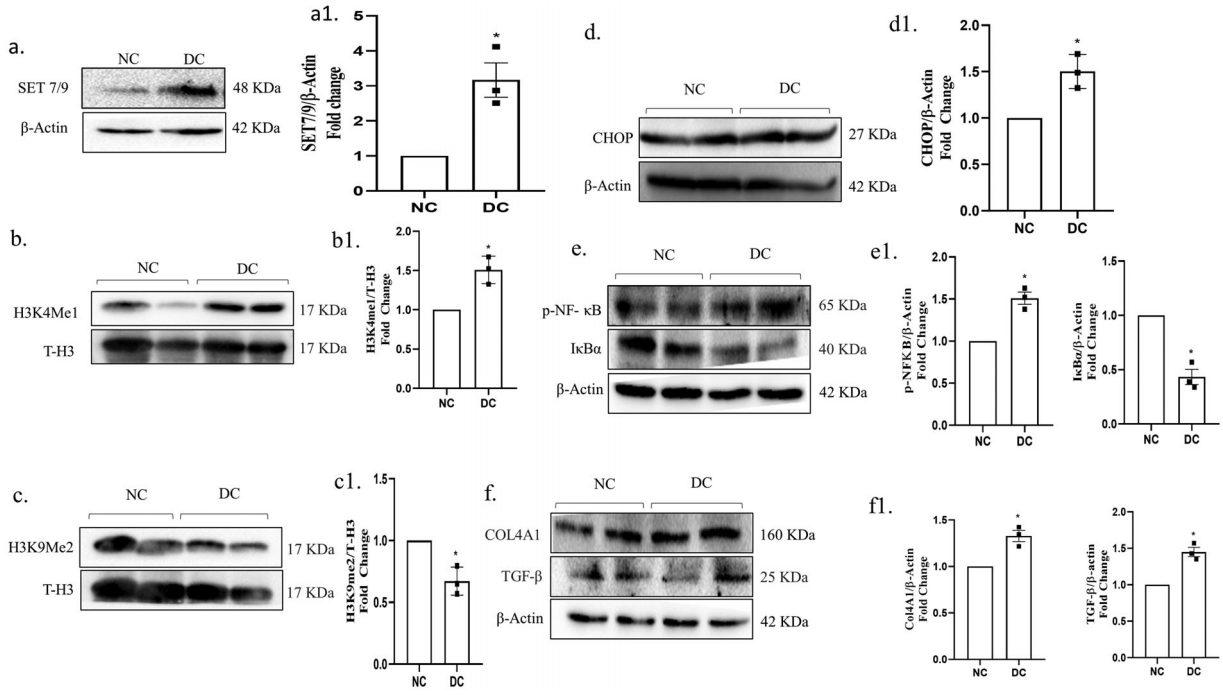

Figure 1. Diabetic kidney disease increases the protein expression of SET7/9, H3K4Me1 and, pro-inflammatory and profibrotic markers and decreases the H3K9Me2 expression in the kidney of rats. (a-f) Representatives immunoblot images and scattered-bar plots, (a1-f1) depicted fold change in protein expressions of SET7/9, H3K4Me1, H3K9Me2, CHOP, p-NF-κB(S-536), IκBα, p-IκBα, TGF-β, Smad7, Col4A1 when compared with NC rats' kidney, respectively. β-actin and TH3 was used as a loading control to normalise protein amount. All the values are represented as mean ± SEM; $n = 3$. [(*) $p < .05$ vs NC]. t-test was used to evaluate the significance level amongst the groups.

Table 1. Biochemical and Morphometric parameters of the normal and diabetic animals.

	Biochemical Parameters				Morphometric parameters		
Group	PGL (mmol/l)	PCr (mg/dl)	Scr (mg/dl)	BUN (mg/dl)	BW (gm)	KW (gm)	(KW/BW) × 100
NC	5.16 ± 0.49	1.1 ± 0.12	0.9 ± 0.03	21.36 ± 1.2	280 ± 12	0.74 ± 0.04	0.264 ± 0.016
DC	21.14 ± 1.12*	2.1 ± 0.17*	1.89 ± 0.12*	48.19 ± 3.1*	160 ± 6*	1.0 ± 0.03*	0.625 ± 0.038*

All the values are represented as mean ± SEM ($n = 8$). PGL: plasma glucose level; PCr: plasma creatinine; Scr: serum creatinine; BUN: blood urea nitrogen; NC: normal control; DC diabetic control; BW: body weight; KW: kidney weight. *$p < 0.05$ vs. NC.

Table 2. Metabolic characteristics and creatinine clearance of normal and diabetic animals.

Group	Water consumed (ml/day)	Total urine volume (ml/day)	Urine Creatinine (mg/dl)	Creatinine clearance (mL/min)
NC	35 ± 5.45	20.5 ± 3.25	55.87	0.88 ± 0.13
DC	185 ± 8.80*	50.3 ± 4.50*	19.54*	0.36 ± 0.09*

All the values are represented as mean ± SEM ($n = 8$). NC: normal control; DC: diabetic control; *$P < .05$ vs. NC.

Incubation with cyproheptadine reduced the expression of H3K4Me1 in NRK-52E cells

Previous studies have established the association between increased expression of H3K4Me1 and transcriptional activation of TGF-β signalling (Sasaki *et al.* 2016). SET7/9 is known to catalyse the activation of H3K4Me1. So, we evaluated the effect of Cyproheptadine on the expression of H3K4Me1 in NRK-52E cells. The enhancement in the active chromatin mark (H3K4Me1) due to persistent hyperglycaemia increases the inflammatory and fibrotic signalling. We found that Cyproheptadine treatment significantly suppressed the expression of H3K4Me1 (Figure 2(a)).

Cyproheptadine treatment curbed the NF-κB signalling pathway activation in NRK-52E cells

Persistent hyperglycaemia and development of DKD in the animals could be attributed to activation of the pro-inflammatory NF-κB signalling pathway (Foresto-Neto *et al.* 2020). We also observed an enhanced level of p-NF-κB and a reduced level of IκBα in the kidneys of the type 1 diabetic animals (Figure 1(e)). Furthermore, NRK-52E cells upon exposure to high-glucose for 48 h also showed elevated levels of p-NF-κB and p- IKKα/β and p-IκBα (Figure 2(b)). The cells when treated with Cyproheptadine, showed marked inhibition of the NF-κB signalling as observed by reduced p-NF-κB and p-IKKα/β and p-IκBα (Figure 2(b)). These results indicate that treatment with Cyproheptadine reduces inflammation.

Cyproheptadine exposure significantly abrogated the profibrotic TGF-β signalling in NRK-52E cells

TGF-β signalling is central to the development of renal fibrosis, which is the outcome of most chronic kidney diseases including DKD (Sasaki *et al.* 2016). In the kidney of the type

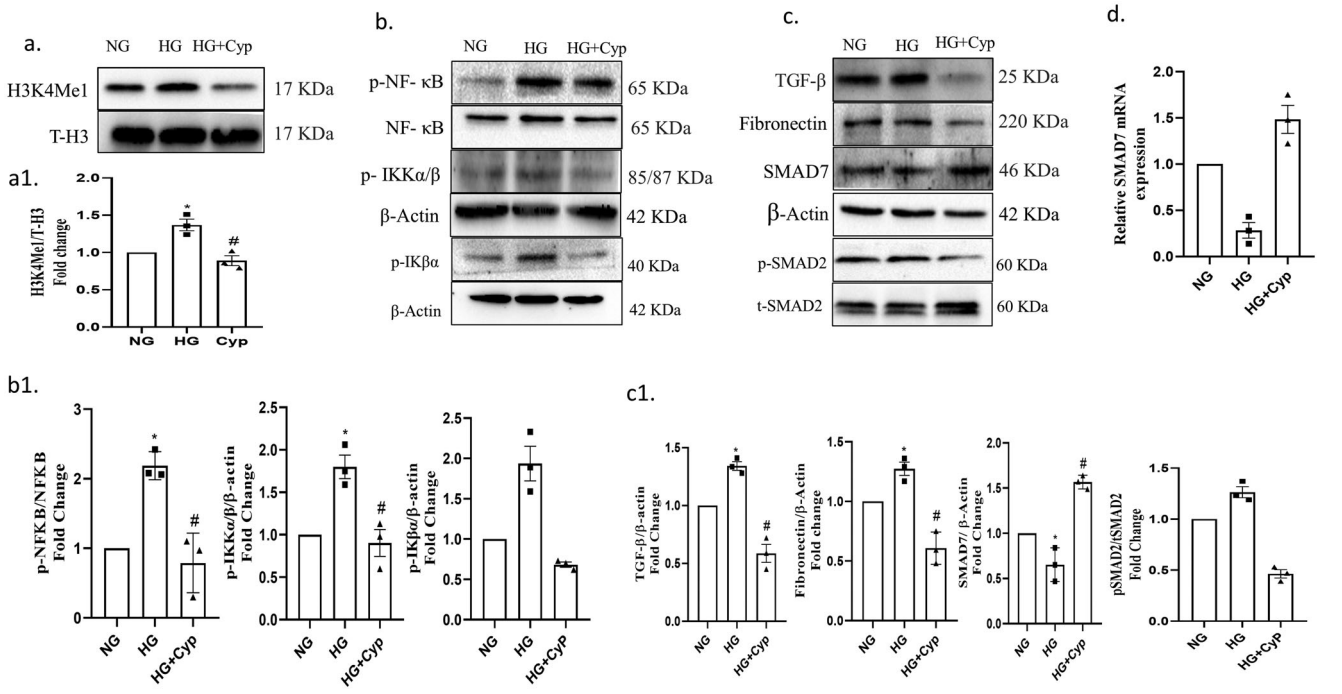

Figure 2. Cyproheptadine treatment normalised histone methylation and ameliorated inflammation and fibrosis. (a) Representative western blot images for protein expressions of H3K4Me1 normalised against TH3 in NRK-52E cell lysates. (b-c) Represents, immunoblots for inflammatory [p-NF-κB (S-536, p-IKβα and p-IKKα/β] and fibrotic (TGF-β, p-SMAD2 and SMAD7) markers. Respective β-actin, NF-κB or p-SMAD2 were used as a loading control. a1-c1) depicted fold change in protein expressions of the immunoblots in fig a-c. All the values are represented as mean ± SEM ($n = 3$). One-way ANOVA with Tukey's multiple comparisons test, where (*) $p < .05$ vs. NG; (#) $p < .05$ vs. HG was used.

1 diabetic animals, TGF-β signalling was dysregulated as evidenced by the increased TGF-β, alpha 1 chain of type IV collagen (Col4A1) and decreased SMAD7 levels (Figure 1(f)). A significant increase in the levels of fibronectin and collagen was also noted. More importantly, Cyproheptadine significantly attenuated the TGF-β signalling in the NRK-52E cells which were shown by reduced TGF-β, pSMAD2 expression and enhanced SMAD7 levels (Figure 2(c)). Increased mRNA expression of SMAD7 in Cyproheptadine treated NRK-52E cells also indicates the reduction in fibrosis (Figure 2(d)).

Cyproheptadine treatment reduced the expression of CHOP and p-eif2α in NRK-52E cells

ER stress has emerged as a decisive factor in the progression of the DKD (Chen *et al.* 2014). The current study also found a significant increase in the ER stress markers such as phospho- eukaryotic initiation factor 2 α (p-eif2α) and C/EBP homologous protein (CHOP) *in-vivo* (Figure 1(d)) and *in-vitro* (Figure 3(a–c)). Interestingly, we observed that Cyproheptadine significantly ameliorated the ER stress compared to the hyperglycaemic group, as observed by the reduced expression of both p-eif2α and CHOP. Similarly, during immunofluorescence assay, the fluorescence intensity of CHOP was significantly higher in the cells incubated with high glucose (30 mM). However, the intensity considerably diminished in the co-incubated cells with high glucose and Cyproheptadine. A significant reduction in the mRNA levels of CHOP was also observed in Cyproheptadine-treated NRK-52E cells (Figure 3(b)). These results indicate that Cyproheptadine treatment significantly attenuates the ER stress and ensuing kidney disease progression possibly by inhibiting the SET7/9.

Cyproheptadine treatment reduced the hyperglycaemia induced cell apoptosis in NRK-52E cells

Cells incubated with HG showed considerable elevation in apoptosis when compared to the those incubated with NG. Interestingly, Cyproheptadine treatment of the cells reduced the apoptosis significantly in comparison to the HG group ($p < .05$) (Figure 4).

Discussion

Persistent hyperglycaemia is a well-known factor in the progression of DKD (Anders *et al.* 2018, Barrera-Chimal and Jaisser 2020). Injury to the renal tubular epithelial cells due to high glucose is important to progression of DKD. Also, hyperglycaemia is known to modulate the epigenetic machinery such as altered histone methylation pattern such as an increase in the active chromatin marks (H3K4Me1/2/3) or decrease in the repressive chromatin marks (H3K9Me2/3) (Lu *et al.* 2021). Previously too, change in HMT levels (SET7/9; SUV39H1; Ezh2 etc.) and resulting histone modifications have been linked to renal fibrosis (Sun *et al.* 2010, Lu *et al.* 2021). This study corroborates the role of hyperglycaemia in epigenetic alteration, where it led to an increase in SET7/9 elevation and consequential increase in active chromatin mark H3K4Me1 and decreased repressive mark H3K9Me2. The study also showed that inhibition of SET7/9 led to amelioration in ER stress, inflammation, and renal fibrosis (Figure 4).

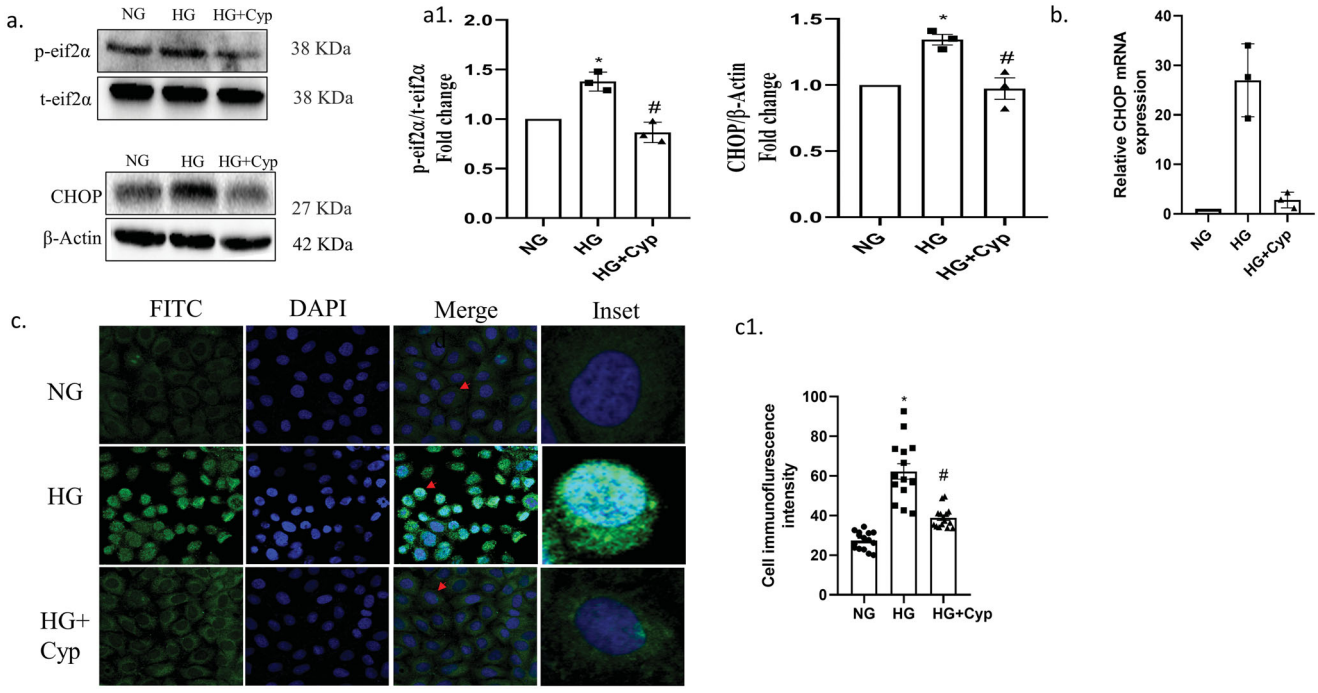

Figure 3. Incubation with Cyproheptadine lead to reduced expression of CHOP and p-eif2α in NRK-52E cells. (a) Representative western blot images for protein expressions of p-eif2α and CHOP. t-eif2α and β-actin were used as loading controls. a1) depicted fold change in protein expressions of the immunoblots of p-eif2α and CHOP. (b) Represents the change in relative mRNA expression of CHOP. (c) Immunofluorescence of CHOP exposed to normal glucose, high glucose and high glucose with Cyproheptadine. CHOP is shown by green fluorescence, and cell nuclei stained with 4′,6-diamidino-2-phenylindole by blue fluorescence. All the values are represented as mean ± SEM ($n = 3$). c1) depicts the change in the relative quantification of CHOP by cells based on their fluorescent intensity. One-way ANOVA with Tukey's multiple comparisons test was used for statistical analysis, where (*) $p < .05$ vs. NG; (#) $p < .05$ vs. HG.

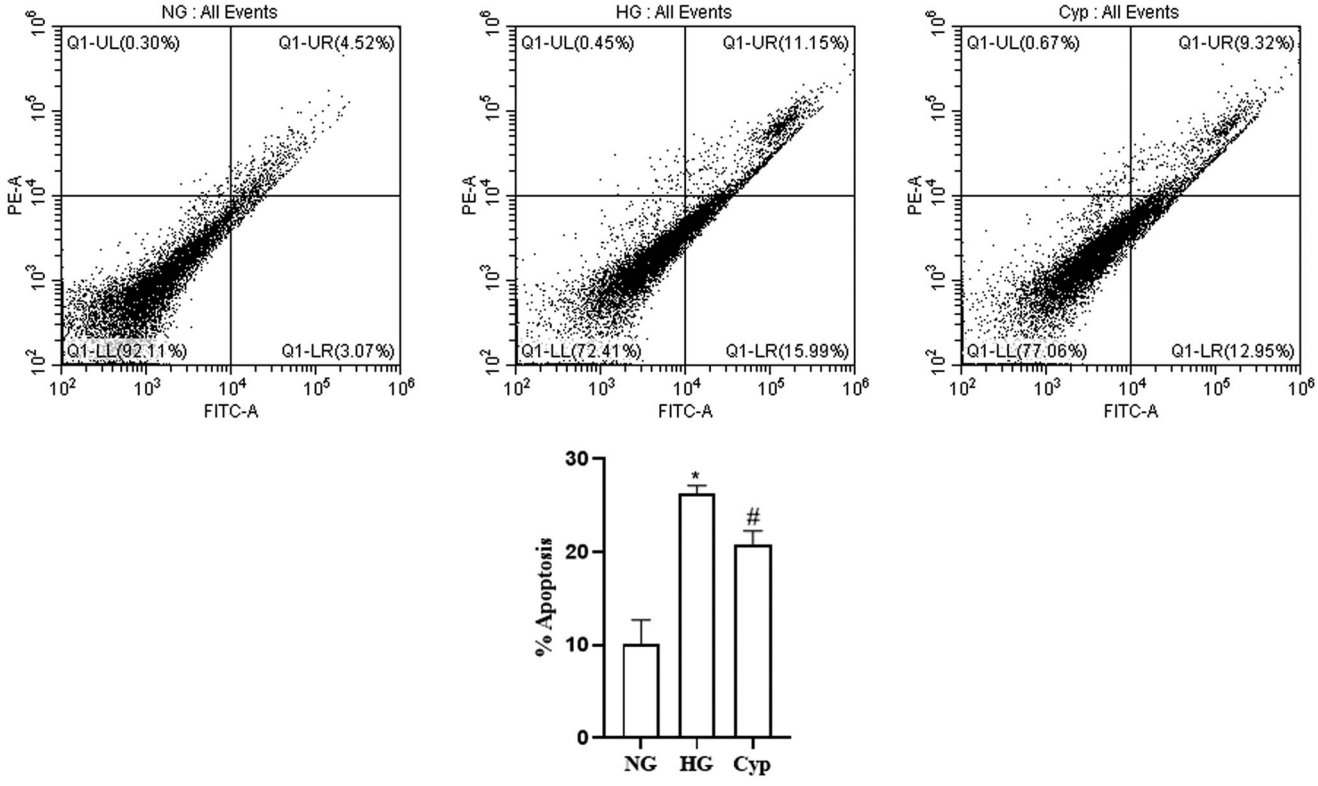

Figure 4. Cyproheptadine treatment significantly reduced percentage apoptotic cells. In the graph Q2 shows the cells in early apoptotic stage and Q3 the late apoptotic stage of the cells. Percentage apoptotic cells is the sum of Q2 and Q3. The percentage apoptotic cells have significantly increased in HG group when compared to NG group which reduced on treatment with Cyproheptadine. All the values are represented as mean ± SEM ($n = 3$). One-way ANOVA with Tukey's multiple comparisons test was used for statistical analysis, where (*) $p < .05$ vs. NG; (#) $p < .05$ vs. HG.

Moreover, we have shown for the first time that Cyproheptadine could be a potential therapeutic entity against DKD. Cyproheptadine, an inhibitor of SET7/9 activity was found to not only reduce the expression of H3K4Me1 but also reduce the ER stress, apoptosis, pro-inflammatory and profibrotic markers in NRK-52E cells thus protecting them.

Cyproheptadine was found to inhibit the SET 7/9 activity in cancer cells (Takemoto *et al*. 2016). SET7/9 is a known initiator of inflammation and fibrosis, renal and extra-renal (Chen *et al*. 2014, Sasaki *et al*. 2016, Tamura *et al*. 2018). Previously, Sasaki *et al*. found that SET7/9 was associated with the development of TGF-β1 associated renal fibrosis and inhibition of the same using Sinefungin ameliorated the renal fibrosis (Sasaki *et al*. 2016). Tamura *et al*. found that SET7/9 has a role in increasing peritoneal fibrosis (Tamura *et al*. 2018). Similar findings regarding the role of SET7/9 in inflammation and ensuing fibrosis were reported by Li *et al* (Li *et al*. 2008). Based on our findings, we hypothesised that Cyproheptadine could be useful against the progression of DKD owing to its SET7/9 inhibitory potential it may protect the NRK-52E cells from hyperglycaemia induced injury.

Type 1 diabetic rats showed significant deterioration in renal functions as evidenced by a significant elevation in BUN levels, plasma creatinine. Type 1 diabetic kidneys showed overexpression of profibrotic (COL4A1, Fibronectin and TGF-β) and pro-inflammatory (p-NF-κB, p-IKKα/β) markers. Moreover, it was found that the expression of SET7/9 is significantly increased in the kidney of type 1 diabetic animals. Further, changes in active and repressive chromatin marks from the histone isolated from type 1 diabetic kidneys corresponded with the previous findings (Chen *et al*. 2014, Sasaki *et al*. 2016). Besides causing epigenetic changes, hyperglycaemia also leads to the generation of ER stress, now recognised as an important mediator in the progression of DKD. ER stress is more pronounced in the tubular portion of the kidney during DKD. Hence, we chose to specifically study the changes in the proximal tubular epithelial cells of the rat kidney (NRK-52E cells).

We observed increased H3K4Me1 in the hyperglycaemic NRK-52E cells. Our results are in corroboration of previous findings where hyperglycaemia has led to an increase in the level of SET7/9 and resulting histone lysine methylation in different cells. Yunlei D reported that upon exposure to high glucose (25 mM), rat glomerular mesangial cells (HBZY-1) indeed increased the expression of SET7/9. They also observed the resulting increase in the H3K4Me1 (Yunlei *et al*. 2018). In another study involving vascular endothelial cells, SET7/9 levels were elevated after incubation under high glucose, again followed by higher levels of H3K4Me1 (Okabe *et al*. 2012). Moreover, we also report that Cyproheptadine significantly suppressed the H3K4Me1 levels in NRK-52E cells. The enhancement in active chromatin marks (K3K4Me1) and decrease in the repressive chromatin mark (H3K9Me2) is responsible for activating the downstream fibrotic and inflammatory signalling cascade.

Further, SET7/9 and H3K4Me1 have been linked to the generation of inflammation. It was found that SET7/9 primarily mediates the hyperglycaemia-associated inflammation via activating the transcription factor NF-κB (Paneni *et al*. 2015). In our study, too, we observed the activation of the said transcription factor as shown by increased expression of p-NF-κB p65. This led to an increase in the pro-inflammatory downstream proteins such as p-IKKα/β. This SET 7/9 mediated inflammatory cascade could potentially activate the renal fibroblast cells leading to fibrosis at the end. As per literature, the presence of inflammatory cells and cytokines is a vital determinant of the mechanism of activation of renal fibroblasts (Kanasaki *et al*. 2013).

Moreover, the role of hyperglycaemia in increasing the activity of TGF-β is well known (Di Paolo *et al*. 1996, Li *et al*. 2003, Hayashida and Schnaper 2004). Precisely, TGF-β signalling could be seen as an important mediator in the progression of renal fibrosis associated with DKD (Chang *et al*. 2016, Zhao *et al*. 2020). We also found an increase in TGF-β both *in-vitro* and in the kidney of type 1 diabetic rats. Along with that, we also observed reduced SMAD7 (a negative regulator of TGF-β) in the cells incubated with high glucose. Earlier, SET7/9 was reported to activate the TGF-β signalling. When we treated the cells with Cyproheptadine, we observed a marked reduction in the TGF-β and elevation in the levels of SMAD7 indicating the reversal of TGF-β signalling by Cyproheptadine via SET7/9 inhibition.

Furthermore, hyperglycaemia and the resulting alteration in TGF-β signalling during DKD also lead to the generation of ER stress (Lee *et al*. 2016). Post ER stress, UPR is induced to counter the same, and CHOP is an important downstream to the same. Studies report that deletion or reduction of CHOP is in fact, beneficial in DKD and CKD (Carlisle *et al*. 2021, Wang 2021). PERK-eIF2α-CHOP signalling has been implicated in the development of diabetes-related fibrosis (Wang *et al*. 2021). In the current study, an increase in the levels of p-eIF2α and CHOP was observed indicating an increase in ER stress. Both hyperglycaemia and SET7/9 have been linked to the generation of ER stress individually. Interestingly, in our study ER stress was found to be reduced on Cyproheptadine treatment as evidenced by reduced CHOP and p-eIF2α expression in NRK-52E cells which could be attributed to its SET7/9 inhibitory potential. Moreover, it is well known that insistent hyperglycaemia induces cell apoptosis which is vital to the progression of kidney damage to fibrosis. Cyproheptadine successfully reduced the percentage cell apoptosis due to hyperglycaemia indicating towards its capability in preventing the progression towards fibrosis.

Conclusion

Our results concur that hyperglycaemia may add on to the progression of DKD by injuring NRK-52E cells via an increase in SET7/9 and ER stress. Moreover, we provided a base for further studies regarding the use of Cyproheptadine, a clinical entity in diabetic conditions to prevent the deterioration of kidney health. We also tested the hypothesis that Cyproheptadine might prevent the damage to NRK-52E cells owing to its SET7/9 inhibitory potential. We revealed that Cyproheptadine successfully attenuated the hyperglycaemia-associated damage to tubular epithelial cells by reducing

H3K4Me1 and ameliorating ER stress and associated inflammation, apoptosis and fibrosis. SET7/9 could prove to be a crucial target for the development of a therapeutic strategy against DKD. Cyproheptadine or the moieties based on it could be developed as a novel SET7/9 inhibitor for clinical use. However, further exploration at the preclinical and clinical levels is warranted.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The authors agree to provide the current study's data on reasonable request.

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ARTICLE

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[1]

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Abstract: Persistent hyperglycemia in type 1 diabetes triggers numerous signaling pathways, which may prove deleterious to the endothelium. As hyperglycemia damages the endothelial layer via multiple signaling pathways, including enhanced oxidative stress, downregulation of angiotensin-converting enzyme 2 signaling, and exacerbation of endoplasmic reticulum (ER) stress, it becomes difficult to prevent injury using monotherapy. Thus, the present study was conceived to evaluate the combined effect of ER 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^{-1} per day, p.o.) or tauroursodeoxycholic acid, sodium salt (200 mg·kg^{-1} per day i.p.), or both for 4 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 with 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.

Key words: endothelial dysfunction, endoplasmic reticulum stress, angiotensin-converting enzyme 2, type 1 diabetes.

Résumé : Dans le diabète de type 1, l'hyperglycémie persistante entraîne le déclenchement de nombreuses voies de signalisation, ce qui pourrait avoir des effets résolument délétères sur l'endothélium. Puisque l'hyperglycémie entraîne des dommages dans la couche endothéliale par l'intermédiaire de nombreuses voies de signalisation, y compris l'augmentation du stress oxydatif, la régulation à la baisse de la signalisation par l'enzyme de conversion de l'angiotensine de type 2, de même que l'exacerbation du stress du réticulum endoplasmique, etc., il devient difficile de prévenir la production de lésions à l'aide d'une monothérapie. Par conséquent, nous avons conçu la présente étude en vue d'évaluer l'effet combiné de l'inhibition du stress du réticulum endoplasmique et de l'activation de l'enzyme de conversion de l'angiotensine de type 2, deux voies de signalisation contribuant de manière très importante au dysfonctionnement endothélial engendré par l'hyperglycémie, dans la prévention du dysfonctionnement endothélial associé avec le diabète de type 1. Pendant quatre semaines, nous avons administré de l'acéturate de diminazène (5 mg·kg^{-1} par jour, p.o.) ou le sel de sodium de l'acide tauroursodéoxycholique (200 mg·kg^{-1} par jour, i.p.), ou encore les deux chez des animaux devenus diabétiques par l'administration de streptozotocine. Nous avons évalué le dysfonctionnement endothélial à l'aide d'une épreuve de vasoréactivité, où nous avons évalué la relaxation provoquée par l'acétylcholine dans des anneaux précontractés par la phényléphrine. Le traitement d'association permettait d'améliorer la relaxation vasculaire par rapport aux témoins diabétiques, et la monothérapie aussi. La vasodilatation s'est améliorée avec le rétablissement des taux de nitrite et la prévention du dépôt de collagène. En outre, nous avons observé une diminution globale du stress oxydatif aortique. Nous en arrivons à la conclusion qu'en inhibant simultanément le stress du réticulum endoplasmique et l'activation de l'enzyme de conversion de l'angiotensine de type 2, les effets délétères de l'hyperglycémie sur l'endothélium s'atténuaient de façon marquée, ce qui pourrait servir de nouvelle stratégie dans la prévention du dysfonctionnement endothélial. [Traduit par la Rédaction]

Mots-clés : dysfonctionnement endothelial, stress du réticulum endoplasmique, enzyme de conversion de l'angiotensine de type 2, diabète de type 1.

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Introduction

Diabetes mellitus leads to different macrovascular complications, which are the major contributors to the morbidity and mortality associated with diabetes (Bjornstad et al. 2018; Varma et al. 2005). This could be primarily attributed to the endothelial dysfunction, which results due to the elevated oxidative stress and endoplasmic reticulum (ER) stress owing to the persistent hyperglycemia (Maamoun et al. 2019; Rajendran et al. 2013). Moreover, endothelial dysfunction is considered an important target to prevent the progression of cardiovascular disorders (Versari et al. 2009). The primary reason for endothelial dysfunction development is the reduced bioavailability of nitric oxide (NO). Several pathophysiological conditions may reduce the NO levels, hyperglycemia being one of them (Chen et al. 2018). Also, persistent hyperglycemia is well known to alter the pressor arm of the renin-angiotensin system and increase the levels of angiotensin-II, which in turn increases the oxidative stress (Lavrentyev et al. 2007; Pueyo et al. 2000) and ER stress in endothelial cells (Liang et al. 2013; Murugan et al. 2015). ER stress could suppress the endothelial NO synthase expression, thus reducing the NO level in the aortic rings obtained from mice (Lau et al. 2018). Studies even suggest a probable mechanistic link between ER stress and oxidative stress being implicated in cardiovascular disorders associated with diabetes, probably because both are important mediators of endothelial dysfunction (Ochoa et al. 2018). Furthermore, hyperglycemia is also known to reduce the angiotensin-converting enzyme 2 (ACE2) levels which form the depressor arm of the renin-angiotensin system and are vasoprotective in nature. ACE2 knockout animals had vascular dysfunction due to reduced NO bioavailability and enhanced oxidative stress (Rabelo et al. 2016). ACE2 and angiotensin (1-7) are emerging as important therapeutic targets to counter the endothelial dysfunction. Direct administration of angiotensin (1-7), 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. As diabetic endothelial dysfunction is of multiple etiology, targeting different signaling pathways simultaneously could be a better approach. Therefore, we hypothesized that simultaneous inhibition of ER stress and activation of ACE2 by novel combination of diminazene aceturate (ACE2 activator) and tauroursodeoxyxcholic 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. (2017). Briefly, type 1 diabetes was induced by injecting a single dose of streptozotocin [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 h 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, which received vehicle treatment; (*ii*) diabetic control (DC) group; (*iii*) DC animals were administered diminazene aceturate (5 mg·kg^{-1} per day, p.o.) (Goru et al. 2017); (*iv*) DC animals were administered TUDCA (200 mg·kg^{-1} per day, i.p.) (Choi et al. 2016); and (*v*) DC animals were treated with a combination of diminazene aceturate (5 mg·kg^{-1} per day, p.o.) and TUDCA (200 mg·kg^{-1} per day, i.p.). The treatments were 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 PGLs. 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 TBA. Briefly, the aortic homogenate was added to freshly made TBA followed by incubation at 95 °C for 1 h. The mixture was further centrifuged, and the supernatant was measured spectrophotometrically at 532 nm for MDA-TBA adduct. The final concentration of MDA was derived from a standard curve and expressed as nM of MDA/mg protein (Kadakol et al. 2017*b*).

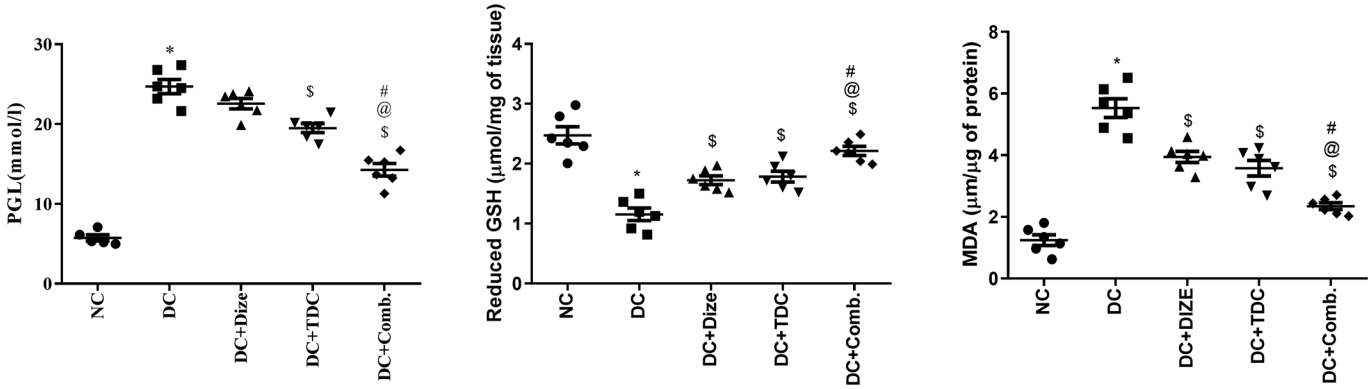
Glutathione (GSH) assay

Reduced GSH 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 GSH. Ellman's reagent was added to the aortic homogenates, followed by an incubation of 15 min. Plates were read at 410 nm, and the concentration of GSH was expressed as μg of GSH/mg protein (Kadakol et al. 2017*b*).

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 min. The buffer in the organ chamber was changed every 15 min. 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 version 7 Pro (AD Instruments, Australia) (Kadakol et al. 2017*a*).

Fig. 1. Effect of different treatments on plasma glucose levels and aortic oxidative stress parameters. All the values are represented as mean ± standard error of the mean; $n = 6$ ($p < 0.05$; * vs. NC; $ vs. DC; @ vs. DC+Dize; # vs. DC+TDC. (*a*) Plasma glucose levels (PGLs); (*b*) reduced glutathione (GSH) levels; (*c*) malondialdehyde (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.



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 min. 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 µmol/mg of protein (Sharma and Gaikwad 2020).

Picro-sirius red (PSR) staining

PSR 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 h. Followed by paraffin embedding. Tissue sections of 6 µm thickness were obtained. Later the sections were stained with PSR dye 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 400× magnification. The images were further analyzed using Image J software for the measurement of %PSR positive area (collagen deposition) (Kadakol et al. 2017*a*).

Statistical analysis

All the experimental values are expressed as mean ± standard error of the mean ($n = 6$). Graph pad PRISM version 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 pD_2 value ($-\log EC_{50}$) 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 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 PGLs, however, diminazene aceturate monotherapy did not reduce the blood glucose levels. Moreover, the combination therapy was found to significantly reduce the blood glucose levels when compared with the DC group as well as monotherapies (Fig. 1*a*).

Diminazene aceturate and TUDCA combination therapy reduced the aortic oxidative stress

The levels of GSH 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. Furthermore, treatment with diminazene aceturate, TUDCA, and their combination for 4 weeks successfully restored the GSH levels and reduced the lipid peroxidation as depicted in Figs. 1*b* and 1*c*. However, the combination therapy brought a significant reduction in the MDA levels as compared with 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 acetylcholine 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 were able to improve the vasorelaxation significantly when compared with 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 DC group aorta in comparison to the NC animals' aorta. Nitrite level restoration

Fig. 2. Concentration dependent relaxation of acetylcholine (Ach) on aortic rings (with intact endothelium) pre-contracted with phenylephrine (PE) obtained from different treatment groups. Tension is expressed as percentage of relaxation on initial contraction with PE. Values are expressed as mean ± standard error of the mean. $p < 0.05$; * vs. NC; $ vs. DC; @ vs. DC+Dize; # vs. DC+TDC. 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.

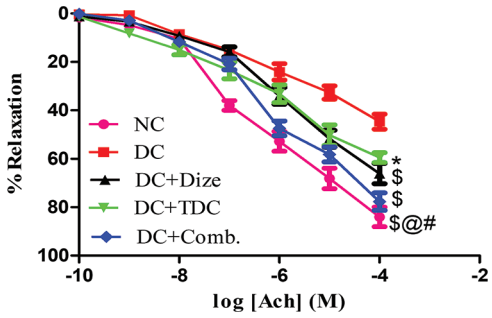


Table 1. The pD_2 (–Log EC_{50}) values of acetylcholine and maximal response to each treatment (E_{max}).

	Acetylcholine	
Groups	pD_2	E_{max} (% 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$@#

Note: $p < 0.05$; * vs. NC; $ vs. DC; @ vs. DC+Dize; # vs. DC+TDC. 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.

was observed post 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).

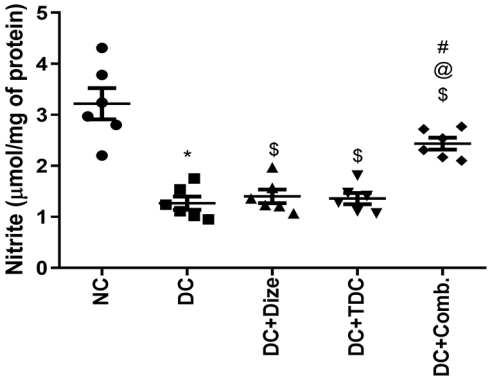
Simultaneous activation of ACE2 and inhibition of ER stress reduced the aortic collagen deposition

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 diminazene aceturate and TUDCA reduced the collagen levels significantly when compared with DC group. 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 monotherapy groups (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

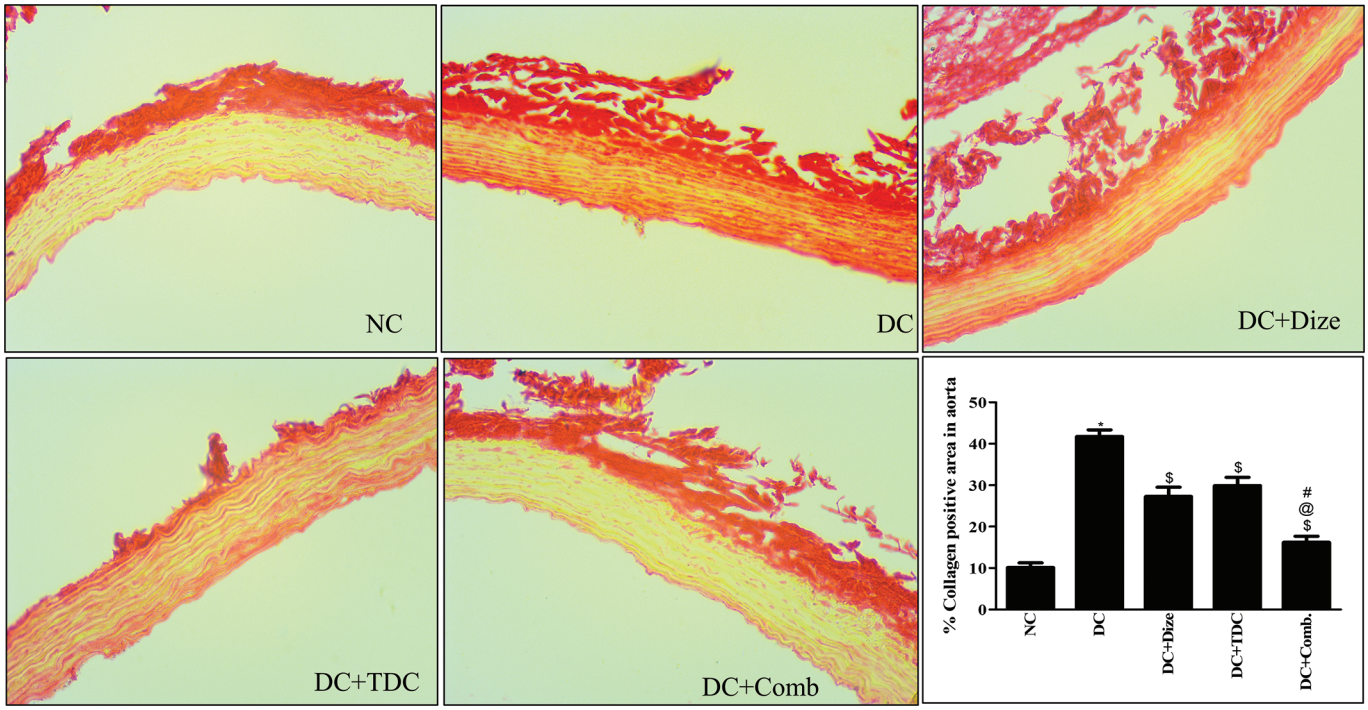
Fig. 3. Effect of different treatments for 4 weeks on aortic nitrite levels. Aortic nitrite levels in different groups. All the values are represented as mean ± standard error of the mean; $n = 6$. $p < 0.05$; * vs. NC; $ vs. DC; @ vs. DC+Dize; # vs. DC+TDC. NC: normal control; DC: diabetic control; 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.



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

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

Fig. 4. Light microscopic pictures illustrating the Picro-sirius red (PSR) staining for collagen deposition (400× magnification) and quantification of percent positive area of collagen. All values are represented as means ± standard error of the mean, $n = 6$. $p < 0.05$; * vs. NC; $ vs. DC; @ vs. DC+Dize; # vs. DC+TDC. 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.



We also found that prolonged hyperglycemia led to the reduction of acetylcholine-induced vasorelaxation in the aorta. This was in line with previous studies where hyperglycemia has led to reduced vasorelaxation (Romero et al. 2008; Sun et al. 2017). Multiple reasons have been proposed for the same, including elevated oxidative stress and ER stress which leads to the damage of endothelium and reduction in the bioavailability of NO (Imanishi et al. 2006). ER stress inhibition could be beneficial in endothelial dysfunction because they have the capacity to restore the bioavailability of NO, an important factor in maintaining vascular homeostasis. In our study, we found that treatment with TUDCA improved the levels of NO in the aorta of diabetic rats. Studies also suggest that ER stress may induce endothelial cell apoptosis and increase oxidative stress, both responsible for endothelial dysfunction. Also, hyperglycemia may lead to elevated oxidative stress due to increased angiotensin-II and reduced ACE2 and angiotensin (1-7) activity. Such an increase in oxidative stress due to elevated angiotensin-II is also known to reduce NO bioavailability. This was supported by our results where ACE2 activation using diminazene aceturate markedly reduced the oxidative stress levels as observed by improved GSH and reduced MDA levels. Due to the interlink pathophysiology of ER stress and oxidative stress, we thought of inhibiting both using a combination of drugs. Consequently, we observed that the combination therapy was better than monotherapies in alleviating oxidative stress and elevating the NO bioavailability. Aortic stiffening is also a major contributor to the loss of the vasorelaxant ability of the aorta (Grigorova et al. 2016). Diabetes leads to the deposition of excess collagen in the aorta. In this study, PSR staining showed excessive collagen in the diabetic rat aorta in comparison to that of the normal rat aorta. Moreover, we observed a significant reduction in the collagen deposition in the animals treated with ACE2 activator and ER stress inhibitor. Hyperglycemia-induced elevation in 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.

Conflict of interest

The authors declare no conflicts of interest.

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