Klotho Regulation as a Novel Therapeutic Strategy against Acute Kidney Injury-Diabetes Comorbidity: Impact of Epigenetic Driven and Epigenetic Independent Reactivation of Endogenous Klotho Expression

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CERTIFICATE

This is to certify that the thesis entitled **"Klotho Regulation as a Novel Therapeutic Strategy against Acute Kidney Injury-Diabetes Comorbidity: Impact of Epigenetic Driven and Epigenetic Independent Reactivation of Endogenous Klotho Expression"** submitted by **Mr. Kale Ajinath Vishwanath** ID No**. 2019PHXF0452P** for the award of Ph.D. of the institute embodies original work done by him under my supervision.

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Acknowledgments

गुरौ न प्राप्यते यत्तन्नान्यत्रापि हि लभ्यते। गुरुप्रसादात सर्वं तु प्राप्नोत्येव न संशयः॥

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Mr. Kale Ajinath Vishwanath

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 III	Angiotensin III
Ang IV	Angiotensin IV
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
BiP	Binding immunoglobulin protein
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
CKD	Chronic kidney disease
CVD	Cardiovascular disease
DM	Diabetic mellitus
Dize	Diminazene aceturate
DNMT1	DNA methyltransferase 1
ECM	Extracellular matrix
eiF2α	Eukaryotic translation-initiation factor 2α
ESKD	End-stage kidney disease
GFR	Glomerular filtration rate
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
HIF-1α	Hypoxia-inducible factor-1α
HMT	Histone methyltransferases
IGF-1	Insulin-like growth factor-1

I/R	Ischemia/reperfusion
IRI	Ischemic renal injury
IL-6	Interleukin-6
LPS	Lipopolysaccharide
LSB	Low salt buffer
MCP-1	Monocyte chemoattractant protein 1
NaZ	Sodium azide
NF-κB	Nuclear *factor* kappa-light-chain-enhancer of activated B cells
NGAL	Neutrophil gelatinase associated lippocalin
NRK52E	Normal rat kidney epithelial cells
PARP	Poly-(ADP-ribose) polymerase
PCr	Plasma creatinine
PERK	Protein Kinase R-like ER Kinase
PGL	Plasma glucose
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1 α
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PTMs	Posttranslational modifications
qRT-PCR	Quantitative real-time polymerase chain reaction
RAAS	Renin-angiotensin aldosterone system
rhACE2	Recombinant human ACE2
ROS	Reactive oxygen species
SAHA	Suberoylanilide hydroxamic acid
TGF-β	Transforming growth factor-β
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
TSA	TSA
TUDCA	Tauroursodeoxycholic acid
uKIM-1	Urinary Kidney injury molecule-1
UUO	Unilateral ureteral obstruction

Abstract

Background

Acute kidney injury (AKI) is a clinical syndrome, highly prevalent in hospitalized and comorbid patients having diabetes, and cardiac diseases, with a substantial mortality rate. The pathogenesis of AKI is complex and has multifactorial aetiology. Moreover, an existing treatment options are non-specific and symptomatic in nature. Hence, the identification of novel prophylactic therapeutics is an urgent need of the hour for the treatment of AKI.

Klotho is such a promising therapeutic option against AKI. However, the underlying mechanisms of Klotho regulations and different approaches for endogenous restoration were not explored in AKI-diabetes conditions. Thus, we studied the epigenetic and non-epigenetic mechanisms of Klotho regulation and evaluated its therapeutic potential against AKI under type 1 diabetes and non-diabetic conditions. Epigenetic modifications occur at the nuclear or molecular level and alter protein/gene expression without direct interaction with the original DNA sequence. Fascinatingly, the pharmacological modulation of the key epigenetic enzymes can inhibit these epigenetic changes and improve kidney disease outcomes. Hence, in one of the objectives, we aimed to study the role of kidney-specific histone deacetylase (HDACs 1,2,3 and 6) in Klotho regulation and AKI development under diabetic and non-diabetic conditions.

The renin-angiotensin-aldosterone system (RAAS) maintains fluid balance, arterial blood pressure and plays a key role in kidney homeostasis. Primarily, the RAAS components are categorized into conventional and non-conventional. Activation of conventional RAAS leads to sodium reabsorption, vasoconstriction, apoptosis, oxidative stress, activation of pro-fibrotic, inflammatory signalling, and Klotho downregulation in the kidney. Interestingly, the non-conventional arm opposes these effects and hence called as protective arm of the RAAS. However, the association between the non-conventional arm i.e. ACE2/Ang-(1-7) and Klotho was unknown. Hence, we check the effect of ACE2 activation on Klotho regulation and AKI progression using diminazene aceturate (DIZE). Endoplasmic reticulum (ER) stress is common pathomechanism involved in the development of kidney disease and diabetes. But, its relationship with Klotho regulation and AKI progression was unexplored. Thus through this study, we find out the effects of ER stress on Klotho regulation and AKI progression under diabetic and non-diabetic conditions.

Methodology

Streptozotocin was used to induce type 1 diabetes in male Wistar rats. Later, bilateral ischemia-reperfusion injury (IRI) for 20 min. followed by 24 h of reperfusion was performed for the induction of ischemic AKI. Similarly, to mimic with *in-vivo* model of ischemic AKI we used an *in-vitro* model (NRK52E cells with 5.5mM/30mM glucose) of chemical hypoxia using 10mM sodium azide (ATP depletion or severe hypoxia) followed by reperfusion with complete media i.e. hypoxia reperfusion injury (HRI). The different pharmacological interventions were used in three different studies. i) For study 1, a) *In-vivo*: the pan histone deacetylase (HDAC) inhibitor- Trichostatin -A (TSA) (0.5 mg/kg/day, *i.p.*), was dissolved in dimethyl sulfoxide (DMSO), and injected at 16 h and 30 min before the surgery. b) *In-vitro*: NRK52E cells were treated with TSA (500nM), maintained throughout the experiment. ii) For study 2, a) *In-vivo*: ACE2 activator- DIZE (15 mg/kg/day, *p.o.)* was dissolved in saline solution and administered for 2 days and 1 h before IRI. b) *In-vitro*: NRK52E cells were treated with DIZE (100 μM) maintained throughout the experiment. iii) For study 3, a) *In-vivo*: ER stress inhibitor- tauroursodeoxycholic acid (TUDCA) (400 mg/kg/day, *p.o.*) was dissolved in saline solution and administered for 3 days and 1 h before IRI. b) *In-vitro*: NRK52E cells were treated TUDCA (800 μM) and/or Tunicamycin (2 μg/ml) maintained throughout the experiment.

To assess the effects of these pharmacological interventions on Klotho expression and AKI progression, various experiments were performed using the *in-vitro* and *in-vivo* samples. To evaluate the expression of AKI and type 1 diabetes biomarkers biochemical analysis was performed using the various reagent and ELISA kits. To check the morphological alterations in ischemic kidney, H&E staining was performed. Further immunohistochemistry and western blotting were conducted for checking the kidney-specific expression of various proteins. To quantify the expression of mRNA, RT-PCR was used. For *in-vitro* related studies, XTT assay was used to study the cytotoxicity and determine the safe dose of drugs. Additionally, flow cytometry analysis was utilized for apoptosis estimation. Other experiments like, western blotting, RT-PCR, Klotho siRNA transfection, etc. were also performed.

Results

Overall results of the study suggest that type 1 diabetes/hyperglycemia significantly exaggerated IRI/HRI-induced AKI as evidenced by biochemical and histological results. This clearly indicates that it acts as independent risk factor in AKI development. Extensive loss of endogenous Klotho (plasma and tubule specific) could be the connecting link behind diabetes exaggerated AKI. Through the first study, we also observed a significant increase in expressions of kidney-specific HDACs (1,2,3,6), apoptotic markers such as c-PARP, caspases and inflammatory proteins like MCP-1, p-IKK-α/β, p-NF-κB and TNF-α (mRNA). Moreover, the levels of endogenous Klotho, H3K9Ac and H3K27Ac proteins were significantly decreased in AKI. However, HDACs inhibition (using TSA) showed reno-protection via restoring the endogenous Klotho loss, increasing the expression of H3K9Ac and H3K27Ac proteins and prevention of inflammation and apoptosis. In the second study, we further confirmed that systemic and renal Klotho deficiency is a novel hallmark of AKI. Additionally, ACE2 is a protective component of the RAAS, and its inhibition/deficiency leads to inflammation, apoptosis, Klotho downregulation, and thus AKI development. Importantly, ACE2 played an important role in Klotho upregulation, which might be act as an intermediate for ACE2-mediated reno-protection. In conclusion, the ACE2 activator i.e. DIZE, restored endogenous ACE2-Ang-(1-7)-Klotho level, inhibited apoptosis and inflammation, and ameliorated AKI under diabetic and non-diabetic conditions. Hence, targeting ACE2-Ang-(1-7)-Klotho axis may prove a novel therapeutic strategy against AKI. In third study, we demonstrated that the expression of AKI biomarkers, ER stress markers such as (R/PKR-like ER kinase (PERK), binding immunoglobulin binding protein (BiP), and eukaryotic initiation factor-2 (eIF2α), were increased after the IRI/HRI. Increased ER stress was associated with apoptosis induction, as depicted by increased levels of renal apoptotic markers such as PARP and caspase-7 and decreased tubular Klotho expression. However, under diabetic conditions, the ER stress and apoptosis were more intense with further Klotho downregulation. Treatment with TUDCA inhibited the ER stress and apoptosis as depicted by decreased expression of associated markers, and restored endogenous Klotho levels which eventually ameliorated AKI under diabetic and non-diabetic conditions.

Conclusion

Our study demonstrated that type 1 diabetes/hyperglycemic condition is an independent risk factor for AKI development in which endogenous Klotho loss could be the possible connecting link. Interestingly, Klotho is a promising therapeutic target against the AKI-diabetes comorbidity wherein its endogenous expression can be restored by targeting epigenetic and non-epigenetic mechanisms. Pharmacological interventions like TSA- a HDAC inhibitor, DIZE- a ACE2 activator, and TUDCA- an ER tress inhibitor produces reno-protection in AKI-diabetes comorbidity via increasing the endogenous level of Klotho. This study strongly recommends Klotho as a novel and promising therapeutic target against AKI under diabetic and non-diabetic conditions. However, to further validate and translate these outcomes at a clinical level, more extensive preclinical and pilot clinical studies are need to be performed.

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1. Introduction

Acute kidney injury (AKI) is a severe and common clinical complication among comorbid and hospitalized patients (Abebe et al., 2021; dward D. Siew1, 2021). AKI results in an abrupt change in kidney structure and a significant reduction in its functions. Complete AKI recovery is uncommon, wherein such patients are found more prone to AKI-chronic kidney disease (CKD) transition and progression towards end-stage kidney disease (ESKD). The lack of early potential biomarkers, and prophylactic or target-specific therapies, ensue in a continuous upsurge in AKI-associated morbidity and mortality rates. Importantly, these rates are always higher in diabetic patients compared to non-diabetic ones (Hapca et al., 2021). However, the mechanisms underlying diabetes-aggravated AKI remain unexplored. The etiology and pathophysiology of AKI are multifactorial and complex. AKI can be induced by sepsis, toxins, infections, or surgery wherein the ischemic form of AKI is the most common (Dong et al., 2019).

Klotho is well known antiaging protein that possesses pleiotropic functions in the body (Christov et al., 2019). It has been in the limelight of research for the last two decades. Klotho is a transmembrane protein with a large extracellular domain containing two internal repeats, KL1 and KL2. Membrane Klotho can be cleaved off by metalloproteases such as ADAM10 and ADAM 17 into its functionally active circulating form, i.e. soluble Klotho (Javier A. Neyra, 2020). Further, Klotho is expressed in different forms: α-, β-, and γ-Klotho with distinct expression patterns and functions in the body. Here, we have studied α-Klotho, further referred to as "Klotho". The large piece of evidence suggests that Klotho has diagnostic and therapeutic potential against various forms of kidney diseases. However, the underlying mechanisms of Klotho regulations and different approaches to its endogenous restoration against AKI are not explored enough.

Epigenetic modifications are changes that occur at the nuclear or molecular level and that alter gene expression without direct interaction with the original DNA sequence (Fontecha-Barriuso et al., 2018). Such epigenetic modifications include DNA methylation, histone modification, and a change in miRNA expressions, which eventually results in a modification of genes/proteins and the induction of disease (Morgado-Pascual et al., 2018). For example, aberrant DNA methyl transferase (DNMT-1/3a), histone deacetylase activities (HDACs-1,2,3,6,8), and altered miRNA expressions (MALAT1, MEG3, MEAT1) are associated with

Klotho modulation and the progression of kidney diseases (Xia and Cao, 2021). Fascinatingly, the pharmacological modulation of the key epigenetic enzymes can inhibit these epigenetic changes and improve kidney disease outcomes (Bikbov et al., 2020). ***Hence, considering these findings, in first objectives, we aimed to study the role of kidney-specific HDACs (1,2,3 and 6) in Klotho regulation and AKI development under diabetic and non-diabetic conditions. We also studied the AKI-interlinked pathological signaling mechanisms, such as apoptosis, inflammation, and their connotation with Klotho regulation in AKI-diabetic conditions.***

The renin-angiotensin aldosterone system (RAAS) regulates fluid balance and arterial blood pressure (Sharma et al., 2019). Primarily, three major hormones aldosterone, renin and angiotensin-II constitute the RAAS. More importantly, the kidney harbours most of the RAAS components such as protein- renin, enzymes- angiotensin-converting enzyme (ACE), ACE2, receptors like angiotensin II type 1 receptor (AT1R) and AT2R, peptides- angiotensin II (Ang II), Ang-III, Ang-IV, Ang 1-7, and aldosterone (Sharma et al., 2019). Importantly, the kidney is the only site to convert pro-renin to renin, which is the starting point of the cascade events and hence targeting RAAS is a current therapeutic strategy to treat kidney diseases (Hsu and Tain, 2021). Ang-II is a major peptide of RAAS, generated after the cleavage of Ang-I by ACE or directly from angiotensinogen by other proteolytic enzymes such as tonine, cathepsin, elastase or chymostatin-sensitive Ang-II-generating enzyme (Hsu and Tain, 2021). Recently, few studies also explored the functions and mechanisms of novel RAAS components such as angioprotectin, angiotensin A, alamandine and ang 1-9 in kidney diseases (Simões E Silva et al., 2021). The latest study confirmed that an enzyme pridoxal-5'-phosphate metabolizes ang-II into a novel peptide angioprotectin, which antagonizes the contractile effects of Ang-II (Michaela Lellig, 2021). However, alamandine is produced from the hydrolysis of Ang 1-7 by aspartate decarboxylase or enzymatic cleavage of angiotensin-A by ACE2 enzyme (Simões E Silva et al., 2021). Nonetheless, the pathophysiological role of these components in kidney diseases is not explored enough clinically. Primarily, the components of RAAS are broadly classified into two major arms: the conventional/pressor arm and the non-conventional/depressor arm (Sharma et al., 2019). The conventional arm consists of- peptide Ang II, ACE and AT1R which under the pathophysiological conditions promote sodium reabsorption, vasoconstriction, apoptosis, oxidative stress, pro-fibrotic, and inflammatory

signalling in the kidney (Simoes et al., 2021). However, the non-conventional arm also called the protective arm of the RAAS, is composed of Ang 1-7/ACE2/Mas receptor axis, which opposes the activity of the pressor arm and thus its activation shows beneficial effects during kidney disease (Kuriakose et al., 2021). Additionally, pressor arm activation triggers Klotho downregulation, while RAAS blockers (ACEi/ARBs) inhibit the pressor arm and also restores the Klotho level in patients with diabetic kidney disease (Karalliedde et al., 2013). Klotho and the RAAS components are intricately connected, demonstrated by mouse models of remnant kidney, Adriamycin nephropathy, and the unilateral ureteral obstruction model of hypertension (CKD) (Cheng, e al., 2018). Interestingly, external Klotho supplementation abolishes pressor arm activation as evidenced by *in-vitro* and *in-vivo* studies. Remarkably, recent preclinical studies have observed that non-conventional RAAS upregulates and activates Klotho in endothelial cells and related vascular complications, demonstrating significant improvements in their functional and structural characteristics. However, the role of non-conventional RAAS in Klotho regulation and AKI development is unknown. ***Thus, in second objective, we hypothesized that during the AKI progression under normal and diabetic conditions, the reduced endogenous Klotho level and non-conventional arm of the RAAS may have close association and thus targeting them may prove a novel therapeutic strategy against AKI.***

The endoplasmic reticulum (ER) is the primary site for protein synthesis, folding, processing, and transport and regulates proteostasis (Almanza et al., 2019). ER is also responsible for lipid synthesis and storage, $Ca2^{+}$ release, and insulin signaling. However, perturbations in cells or an increased burden on ER results in the disruption of ER proteostasis. It leads to an accumulation of unfolded and misfolded proteins in the ER, which activates the unfolded protein response (UPR) (Sankrityayan et al., 2019a). UPR activation is the primary response to ER stress that acts as an adaptive mechanism and/or sensor monitoring system against the ER workload. The UPR mitigates ER stress by inhibiting the global synthesis of new proteins, increasing the degradation of misfolded proteins, or improving the synthesis and folding capacity of the ER (Sankrityayan et al., 2019a). However, under the persistent ER stress conditions, the UPR sensors such as (R/PKR-like ER kinase (PERK), inositol-requiring enzyme 1α (IRE1α), and activating transcription Factor 6 (ATF6) are released from binding immunoglobulin binding protein (BiP) and tries to culminate in ER stress via different

mechanisms, such as apoptosis, autophagy, and inflammation (Almanza et al., 2019). Recent studies have also demonstrated that IRI induces excessive or persistent ER stress in tubular cells of the kidney. Author Noh M.R. et. al. showed that IRI led to an increase in the expression of CHOP protein that later on induced tubular apoptosis, and an injury (Noh et al., 2015). However, CHOP knockout was seen with reduced apoptosis and amelioration in kidney functions (Noh et al., 2015). In another study, the expressions of other ER stress markers- IRE1α, XBP-1, and GRP78 were elevated in the IRI model of AKI (Ding et al., 2020). Cumulatively, it indicates that IRI may cause persistent ER stress which disrupts protein homeostasis and induces cell death in the kidney during the progression of AKI. This is also one of the reasons for the AKI-CKD transition (Habshi et al., 2022). However, the relationship between IRI-induced AKI and ER stress under diabetic conditions remains elusive. Accumulating evidence suggests the key role of ER stress in the pathogenesis of type 2 diabetes. Diabetes is known to promote the accumulation of fatty acids, advanced glycation end-products, and misfolded and non-degraded proteins in the ER which eventually activates the UPR or ER stress (De Blasio et al., 2017). Also, in response to high glucose, cells demand more insulin in the early stage, increasing the burden on the ER to produce more insulin leading to ER stress in type 1 diabetes. There are sets of results that have demonstrated that ER stress inhibition has been found to be ameliorative in diabetes and kidney diseases, wherein the underlying mechanisms have not been adequately explored (Bronczek et al., 2019). ***Also, the effect of ER stress and its inhibition on Klotho regulation in IRI-induced AKI under diabetic/hyperglycemic conditions remains a matter of investigation.***

Thus, in third objective, we have explored the epigenetic and non-epigenetic mechanisms of Klotho regulation, evaluated its therapeutic potential, and finally, studied the ways out for its endogenous restoration using the different pharmacological interventions against IRI/HRI-induced AKI under diabetic and non-diabetic conditions.

2. Review of literature

2.1 Acute kidney injury: a clinical syndrome

AKI is a rapid decline or loss of kidney functions (Makris and Spanou, 2016). Risk, Injury, and Failure; and Loss (RIFLE) criteria apprise that, AKI is not just renal failure but also includes the necessity of renal replacement therapy (Lin and Chen, 2012). From the clinical and prognostic perspective, AKI is detrimental health complications and remains the imperative fundamental problem with a consistent rise in morbidity and mortality rates across the globe. In a cohort study conducted on 185760 hospitalizations, stage the total percentage of AKI was ~22 %. The mortality rate (in-hospitalized) was stage dependent wherein for stage 1 AKI it was 5.1%, for stage 2 AKI it was 13.7% and for stage 3 it was 24.8% (Khadzhynov et al., 2019). Similar to this, in another study out of 2473 hospitalized patients, 1557 patients experienced AKI (Rey et al., 2022). Elderly individuals and comorbid patients with the presence of CKD, cardiovascular disease, immunocompromised, or anemia are more susceptible to AKI (Farooqi and Dickhout, 2016). Moreover, morbidity and mortality rate of AKI is very high in diabetic patients, which stipulate that hyperglycemia may act as an independent risk factor for AKI.

The etiology and pathogenesis of AKI are complex. Multiple factors, including obstruction in kidney blood flow, accumulation of urine in the kidney, fluid loss, bacterial or viral infections, nephrotoxins, sepsis, autoimmune conditions, or drug-induced injury, can cause AKI (Andrew S, et al., 2017). However, an ischemic form of AKI is the leading cause of death. It mainly occurs in clinical settings wherein patients undergo surgeries such as vascular, hepatic, cardiac, and kidney transplantation (Han and Lee, 2019). During these procedures, sudden obstruction in blood flow followed by reperfusion occurs, resulting in an imbalance in oxygen and nutrient supply and induction of the body's adaptive mechanisms (Han and Lee, 2019). This originates in initiating various cellular pathways to maintain the hemodynamics of the kidney that produce inflammatory cytokines and apoptotic markers, eventually causing cell necrosis and death. The ischemic form of AKI primarily affects the tubular epithelium by damaging its structure and, thus, functions (Patschan and Müller, 2015). Specifically, ischemia obstruction in blood flow leads to ATP depletion and increased reactive oxygen species production, which increases the calcium load in the kidney cells. The increased load activates the enzymes such as proteases, caspases, and phospholipases, further degrading the

cell membrane and cell-specific proteins and thus disturbing associated functions. Due to tubular injury, electrolyte, and fluid balance disturbance occurs that further disturbing kidney homeostasis.

Clinically, AKI has been divided into different phases based on its progress (Basile et al., 2012). The first phase is initiation phase manifested by sudden fall in blood supply to the kidney results in nutrient, oxygen and ATP depletion. This causes injury and thus dysfunction of kidney cells characterized by inflammation and apoptosis. This phase mimics IRI-induced AKI models (Basile et al., 2012). The second phase also called as extension phase, is when the body's compensatory mechanisms get activated, wherein the tubular cells function excessively by polyploidization and hypertrophy mechanisms (Sharma et al., 2019a). This phase is characterized by persistent inflammation and necrosis in the corticomedullary junction of the kidney and a gradual decrease in the glomerular filtration rate. Later on, it enters into the maintenance phase also known as the reorganization phase in which cell migration, severe apoptosis, and irreversible loss of nephron are observed (Sharma et al., 2019a). At this stage, if AKI remains untreated, the other parts of the kidney, including the cortex, glomeruli, and the vascular network, get affected, leading towards CKD and ESKD and thus eventually resulting in kidney failure (Ikizler et al., 2021; Patschan and Müller, 2015).

AKI is manifested by tubular injury and thus the reduction in the level of proteins/genes expressed by tubular cells. Klotho is one of such proteins highly expressed by the tubular cells of the kidney (Buchanan et al., 2020). In a few clinical and preclinical reports significant decrease in Klotho level is reported. Interestingly, in normal individuals and AKI survivors high Klotho level is noted. This indicates the close association between Klotho and AKI pathogenesis. However, its mechanisms of regulation, role and therapeutic potential against AKI and other kidney diseases is inadequately explored.

2.2 Klotho regulation and its functions in normal kidney homeostasis

In the kidney, Klotho is primarily expressed in the distal convoluted tubules and to a lesser extent in proximal convoluted tubules (Kim et al., 2017). During homeostasis Klotho is mainly regulated through FGF-23. FGF-23 is an osteocyte-derived growth factor regulating mineral metabolism. FGF23 acts via its co-receptor α-Klotho, as Klotho and FGF23 null mice

share nearly identical phenotypes consistent in disrupted FGF23 signaling presenting as hypercalcemia, hyperphosphatemia, and elevated levels of vitamin D (Olauson et al., 2012). Hypercalcemia contributes to the onset of nephrogenic diabetes insipidus with ensuing polyuria, volume depletion, and acute renal failure (Sharon M. Moe, 2008). Hyperphosphatemia is an early indicator of kidney failure and may have the major role in downregulating Klotho expression (Munoz-Castaneda et al., 2017). It also decreases vitamin D levels by inhibiting the conversion of calcidiol 1,25(OH)D into calcitriol 1,25$(OH)_2D_3$, the active form of vitamin D, via inhibition of the activity of 1α-hydroxylase. Hence, Klotho regulates kidney homeostasis through maintaining the balance of calcium, phosphate, and vitamin D. In this manner, it suppresses phosphate absorption, stimulated by 1,25$(OH)_2D_3$ in the intestinal tract via the co-receptor NaPi-IIb, which is inhibited by tKlotho-mediated FGF23. This process involves impeding 1 α -hydroxylase synthesis and inactivation of active hormone via 24-hydroxylase up-regulation, which lowers the 1,25$(OH)_2D_3$ circulating level and phosphate level (Andrukhova et al., 2014; Karpe and Tikoo, 2014). Klotho also upsurges urinary phosphate excretion via diminishing the expression of sodium-phosphate co-transporters (NaPi2a and NaPi2c) (Karpe and Tikoo, 2014) and sodium reabsorption via upregulating the expression of sodium chloride cotransporters in the proximal convoluted tubule (Christov et al., 2019). All these mechanisms favor hypocalcemia but Klotho upturns intestinal calcium absorption by activating signaling cascades involving Erk1/2, SGK-1 and WNK4 via stabilization of transient receptor potential vallinoid-5 channel (TRP V5) in the distal convoluted tubule to maintain calcium phosphate homeostasis (Andrukhova et al., 2014).

Apart from Klotho`s roles in mineral metabolism, Klotho also affects the RAAS (Nishiyama and Kobori, 2018; T Yamagishi 1, 2001) and hence blood pressure, electrolyte balance, hemodynamics, and kidney homeostasis. The RAAS has different components including renin, ang-I, ACE, ang-II, ACE2, ang-(1-7), etc. which are having distinct functions in the body. Increase in Ang-II level and/or activity leads to the activation of certain inflammatory pathways, which ultimately downregulates Klotho and insults the cells of the kidney (Sharma et al., 2019a; Zhou et al., 2015).

2.3 Klotho: a potential diagnostic and therapeutic target against AKI and other forms of the kidney disease

Klotho is a novel renoprotective anti-aging protein available in membrane-bound or soluble form. mKlotho acts as an obligatory receptor for fibroblast growth factors (FGFs) controlling vitamin D and mineral metabolism (Dalton et al., 2017). In contrast, soluble Klotho acts as a circulating mediator and is known to function both in FGF23-dependent and -independent manner. It has pleiotropic activities owing to its regulation of various signaling pathways such as insulin/IGF-1 signaling (Nagasu et al., 2011), transforming growth factor-β1/Smad signaling (Irifuku et al., 2016), Wnt/β-catenin signaling (Dai et al., 2009), NF-ƙB signaling (2013) but the exact mechanisms via which Klotho regulates these signaling pathways is poorly understood (Kim et al., 2017; Lewin and Olgaard, 2015; Lindberg et al., 2014). Klotho is expressed in the brain, pancreas, and other solid organs but shows the highest expression levels in the kidney (Barker et al., 2015). Klotho sustains normal kidney physiology but Klotho regulation also contributes to the progression of kidney disease. Systemic and intra-renal levels of Klotho fall drastically during AKI, kidney fibrosis, diabetic nephropathy (DN), and other forms of chronic kidney disease, etc. Moreover, exogenous supplementation or overexpression of endogenous Klotho attenuates kidney disease. The regulation of endogenous Klotho expression involves epigenetic as well as non-epigenetic mechanisms. Epigenetic modifications such as DNA methylation, post-translational *histone* modifications, miRNAs regulate the change in Klotho expression in kidney disease. Non-epigenetic mechanisms such as ER stress, Wnt signaling, activation of the RAAS, excessive reactive oxygen species and cytokine generation, albumin overload, and PPAR-γ signaling also contribute to Klotho regulation. Evolving evidence highlight the capacity of natural products to regulate Klotho expression in kidney disease. All these preclinical data suggest that Klotho could be a novel biomarker as well as therapeutic target.

2.4 Klotho regulation during the development of AKI and other kidney disease

As mentioned above, Klotho is a multifunctional protein with numerous roles in kidney physiology. Interestingly, in the various glomerular or tubulointerstitial forms of kidney disease the expression of Klotho mRNA and protein decline (Cheng et al., 2010; Haruna et al., 2007). Kidney Klotho levels in such diseases are regulated via different signaling

pathways as summarized in table 1. Several studies have explored the role of Klotho in structural and functional changes in different kidney cells like podocytes, mesangial cells, and glomerular endothelial cells. Podocytes are highly specialized glomerular cells and an injury to them results in albuminuria (Kim et al., 2017). Furthermore, change in the actin cytoskeleton of podocytes is key to different glomerular pathologies. Klotho was found to play a vital role in protection of the glomerular filter by repressing transient receptor potential channel 6 (TRPC6)-mediated $Ca2^{+}$ influx. Moreover, ATP stimulated actin cytoskeletal remodeling and proteinuria were also suppressed by Klotho (Kim et al., 2017). A similarly study reported that Klotho prevents the podocyte injury by inhibiting PKCα/p66SHC. Previously, PKCα is known to induce podocyte injury via different mechanisms during hyperglycemia, which is aggravated by absence of Klotho and ameliorated in its presence (Jiang et al., 2019).

Table 1. Klotho regulation and related signaling pathways in various animal models

#	**Animal model**	**Molecular mechanisms/ signaling pathways**	**Effect on Klotho regulation**	**Ref**
1.	ICR-derived glomerulonephritis (ICGN) mouse model of progressive kidney injury	Mitochondrial oxidative stress, DNA fragmentation and apoptotic pathway	Klotho downregulation	(Haruna et al., 2007)
2.	LPS-induced mouse model of endotoxemia	FGF23-Klotho-vitamin D signalling	Klotho downregulation	(Yoshio Ohyama , 1998)
3.	Cyclosporin-A induced nephropathy	Stimulation of NF-ƙB signaling pathway	Klotho downregulation	(Jin et al., 2017)
4.	Klotho knockdown mice/ Klotho overexpressed transgenic mice	Activation of Insulin-like growth	Klotho downregulation	(Nagasu et al., 2011)

		factor-1 signaling pathway	/ Klotho upregulation	
5.	UUO model of renal fibrogenesissis (mice)	Activation of TGF-β1 signaling pathway	Klotho downregulation	(Hidekazu Sugiura, 2012)
6.	STZ-induced rat model of type 1 diabetes-related nephropathy	Upregulation of ROCK signaling pathways	Klotho downregulation	(Deng et al., 2015)
7.	UUO (uremic) rat model of renal fibrogenesis	Activation of Wnt signaling pathways	Klotho downregulation	(Minoru Satoh, 2012)
8.	db/db mouse model of type 2 diabetes-related nephropathy	Activation of Wnt/β-catenin and RAAS signaling pathways	Klotho downregulation	(Dong et al., 2019)

Two different reports suggested that Klotho attenuates mesangial cell injury in diabetic conditions by regulating early growth response factor 1 (Egr-1). Klotho ameliorated glomerular inflammation and fibrosis by retarding the effect of Egr1/TLR4/mTOR regulatory axis in hyperglycemic conditions (Dong et al., 2019). In addition, Klotho overexpression may arrest mesangial extra-cellular matrix production in hyperglycemic human mesangial cells by suppressing Egr-1 via inhibition of TGF-β1/Smad3 singnaling (Aleksandar Denic, 2017). Glomerular endothelial cell injury is a vital step towards development of DN though the underlying mechanisms are still not clear. A recent study revealed that Klotho overexpression may inhibit hyperglycemia induced glomerular endothelial cell injury by inhibiting Wnt/β-catenin pathway and Klotho knockdown agrravated the injury corroborating the protective role of Klotho (Dong et al., 2019).

As stated earlier, Klotho is abundant in the proximal and distal convulated tubules of the kidney suggesting that changes in the level of Klotho may impact the structural and functional integrity of tubules. This assumption was corroborated by few reports which stated that downregulation and depletion of Klotho by FGF-2 led to tubule-epithelial cell plasticity. Reduced Klotho led to suppression of E-cadherin and overexpression of α-SMA and

fibronectin which led to conversion of tubulo-epithelial phenotype to profibrotic phenotype (Guan et al., 2014). Another study reported that α-Klotho along with the vitamin-D receptor and iron plays a vital role in injury to the proximal convoluted tubule during DN (Dahan et al., 2018). Haruna et al. found that survival rate of mice with immune complex glomerulonephritis (ICGN) without the Klotho transgene was ~30% whereas with the Klotho transgene it was ~70%. ICGN mice without Klotho presented with prominent tubular atrophy, thickened tubular basement membrane, mononuclear cell infiltration along with excessive proteinuria and elevated blood urea nitrogen. They concluded that Klotho did protect the tubular and glomerular pathology by inhibiting mitochondrial oxidative stress (Haruna et al., 2007). The reports discussed above support the renoprotective effect of Klotho and its regulation of glomerular and tubular structure as well as function.

Inflammation and oxidative stress are the major contributors to kidney disease progression (Rapa et al., 2019). Klotho was found to be a contributor to inflammation and oxidative stress in kidney soon after its discovery. This was observed when lipopolysachride administration in rat generated the acute inflammatory stress along with Klotho downregulation (Yoshio Ohyama, 1998). The proteins like NF-ƙB activation triggers inflammation and oxidative stress in the diseased kidney (Dong et al., 2019). Evolving data suggest that Klotho represses NF-ƙB activation and subsequent transcription of proinflammatory genes (Wang and Sun, 2009). Jin, *et al*. explored the role of Klotho in cyclosporine A-induced nephropathy. Cyclosporine A-treated mice were transfected with copies of the Klotho gene using adenovirus as a vector (Jin et al., 2017). Klotho overexpression aggravated cyclosporine A nephropathy via fostering kidney inflammation. The role of NF-ƙB was confirmed by *in-vitro* analysis using RPTC cells cultured with soluble Klotho (Jin et al., 2017).

Insulin-like growth factor-1 (IGF-1) is another pathway which can affect renal hemodynamics via interaction with the RAAS (Bach and Hale, 2015). Klotho modulates the IGF-1 and downstream signaling pathways, which may contribute to its renoprotective properties. The process of auto phosphorylation of insulin/IGF-1 and further downstream signaling proteins such as Akt, Forkhead proteins, tyrosine phosphorylation of insulin receptor substrate and phosphoinositide 3-kinase p85 association with IRS proteins is linked to ROS-induced oxidative stress and kidney damage in mice (Hiroshi Kurosu, 2005; Papaconstantinou, 2009). Similar findings were observed in Klotho knockdown mice, which was reversed upon Klotho

overexpression. Klotho overexpression in transgenic mice inhibits the IGF-1 receptor and activation of insulin/IGF-1 signaling, which prevents oxidative damage as well as hypertrophy of the kidney (Nagasu et al., 2011). This findings were further confirmed by *in-vitro* studies where L6 and H4IIE cells treated with Klotho peptide showed inhibitory action on IGF-1 receptor activation (Hiroshi Kurosu, 2005). Also human proximal tubular cells transfected with a pCAGGS-Klotho plasmid suppressed IGF-1-stimulated NADPH activity and hence stopped tubular cell hypertrophy (Nagasu et al., 2011). Similar Klotho-mediated modulation of IGF-1 signaling and attenuation of associated kidney aberration is reported in few more studies (Bian et al., 2015; Hiroshi Kurosu, 2005).

TGF-β1 is a pleiotropic cytokine expressed by almost all kidney cells where it regulates multiple cellular processes such as angiogenesis, immunomodulation and extracellular matrix formation (ECM) (Li et al., 2020). TGF-β1 signaling is closely related to kidney fibrosis along with elevation of fibrotic markers such as α-smooth muscle actin, fibronectin, and collagen-I together with a downregulation of Klotho (Irifuku et al., 2016). Unilateral ureteral obstruction (UUO) mice, Klotho knockout mice, and TGF-β1-treated human kidney cells exhibit a fibrogenic phenotype along with Klotho mRNA and protein downregulation whereas exposure to recombinant Klotho reversed this effect via inhibition of TGF-β1 and associated downstream signaling effects (Hidekazu Sugiura, 2012). Several reports confirmed the interrelation between Klotho and TGF-β1 signaling in kidney disease (Drew et al., 2017; Takenaka et al., 2020). Along with the TGF-β1 pathway, the ROCK signaling pathways also takes part in the progression of the renal fibrosis as well as renal interstitial volume expansion, which is seen in several animal models (Deng et al., 2015; Hidekazu Sugiura, 2012). The Klotho gene (a recombinant adeno-associated virus (rAAV) carrying mouse Klotho full-length cDNA) delivery in STZ-induced diabetic rats ameliorated kidney hypertrophy as well as fibrosis through repression of the ROCK signaling pathway (Deng et al., 2015). Exogenous Klotho supplementation significantly reduced kidney hypertrophy via repression of the Akt/mTOR signaling. This certainly helps in restoring the normal glomerular filtration process (Takenaka et al., 2020).

Wnt/β-catenin signaling activation plays crucial role in pathogenesis of podocyte injury and proteinuric disorders such as DN and FSGS (Dai et al., 2009; Dong et al., 2019). Phosphateuria activated Wnt/β-catenin signaling cascade and associated reduction in

intrarenal Klotho expression was reported in uremic rats as well as in HEK293 cells (Munoz-Castaneda et al., 2020). Upon treatment with human recombinant Dickkopf-related protein-1, the Wnt pathway inhibitor and calcitriol the expression of Klotho was restored (Munoz-Castaneda et al., 2020). Another study showed that UUO-induced tubulointerstitial fibrosis via Wnt signaling stimulation were absent in Klotho overexpressed transgenic mice when compared with the normal wild type mice (Minoru Satoh, 2012). High glucose-induced endothelial cell injury in human renal glomerular endothelial cells and db/db mouse model of DN found with intermediate proteins of Wnt/β-catenin and RAAS signaling pathways with decline in Klotho level. Also Klotho knockdown by siRNA enhanced the glucose-induced injury in these cells whereas the overexpression of Klotho and injecting the Klotho-overexpressing adenovirus in mice exerted the opposite effect with amelioration of DN (Dong et al., 2019). This notion indicates Klotho may acts as a negative regulator of Wnt/β-catenin signaling which is further described and supported by other suthors as well (Munoz-Castaneda et al., 2020; Zhou et al., 2013).

2.5 Mechanisms of endogenous Klotho regulation in AKI and other kidney disease

The connotation of Klotho modulation and its influence on renal homoeostasis have been well documented as discussed before. Increase in endogenous Klotho expression either with exogenous supplements or via targeting cellular pathways can positively affect therapeutic outcomes in kidney disorders (Bikbov et al., 2020; Neyra and Hu, 2017). Hence, upregulation of endogenous Klotho via epigenetic and epigenetic independent mechanism may be the best strategy to ameliorate kidney abnormalities.

2.5.1 Epigenetic mechanisms of Klotho regulation

Epigenetic modifications are changes that occur at the nuclear or molecular level and that alter gene expression without direct interaction with the original DNA sequence (Morgado-Pascual et al., 2018; Yokoo1, 2019). The most commonly studied epigenetic modifications include DNA methylation, histone modification, and change in miRNA expression (Almanza et al., 2019; Morgado-Pascual et al., 2018). Epigenetic modifications are linked to development and progression of several kidney disorders. Aberrant DNA methyl transferase and histone deacetylase activities and miRNA expressions are associated with kidney diseases and pharmacological modulation of the key epigenetic enzymes can improve kidney disease outcomes. However, changes in the underlying molecular pathways remain frequently

elusive. The emergence of Klotho as a potential renoprotective agent has provided a glimmer of hope. Researchers are studying different factors associated with the decline in Klotho levels as well as pathways which could elevate Klotho levels. Herein we discussed the different epigenetic mechanisms associated with Klotho regulation (Fig. 1).

2.5.1.1 Post-translational histone modifications and Klotho regulation

Histones are the key protein of the chromatin as they compact DNA structure to form nucleosomes and to further guide in the process of gene transcription (Fontecha-Barriuso et al., 2018; Hoeksema et al., 2016). Posttranslational modifications of histones by toxins or environmental factors, i.e. epigenetic modulation, alter protein expressions and activity. Histone modifications include methylation, acetylation, ubiquitination, phosphorylation and others, which are catalyzed by histone acetyl transferases (HAT) and histone deacetylases (HDAC) having opposite functions (Almanza et al., 2019; Fontecha-Barriuso et al., 2018; Ruiz-Andres et al., 2016). These epigenetically driven mechanism of Klotho protein expressions associates with numerous kidney disorders (Bikbov et al., 2020; Jing WANG, 2015).

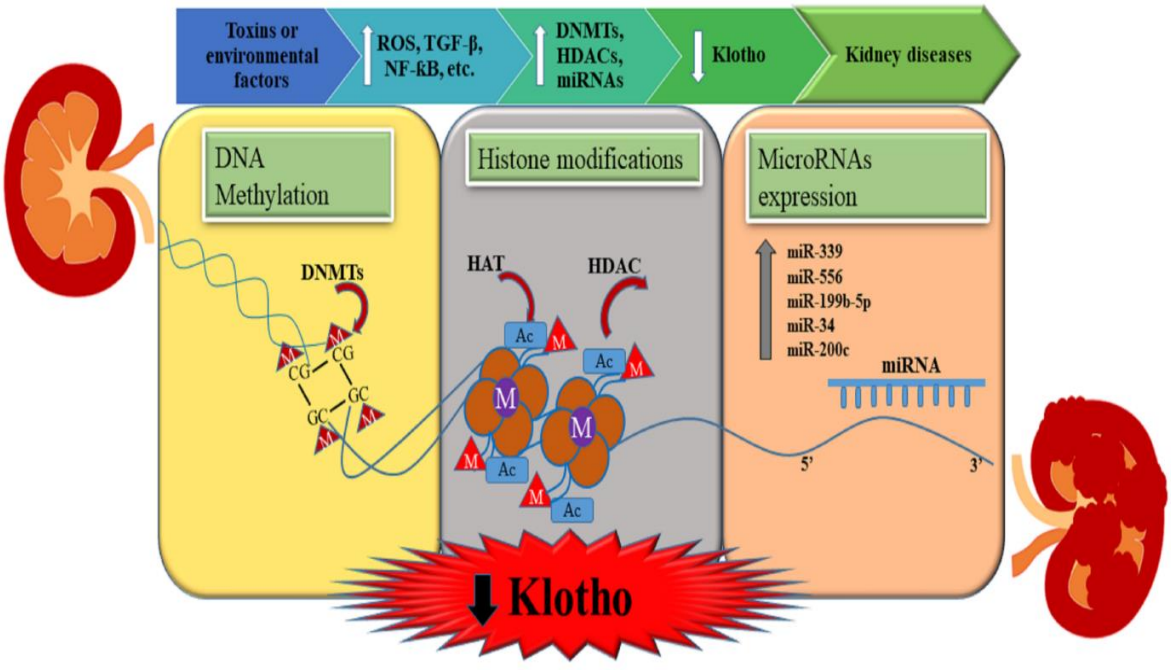


Figure 1: Epigenetic mechanisms of Klotho regulation.

HDACs remove an acetyl group from an amino acid within histones, which unwraps chromatin and downregulates Klotho expressions. Lin, *et al*. used a mouse model of adenine-induced CKD and HK-2 cells to evaluate the effect of HDAC inhibition on Klotho expression.

They found that kidney and systemic Klotho levels were significantly reduced in adenine-fed mice with deterioration in multiple parameters of kidney function. Mice treated with TSA and suberanilohydroxamic acid, showed improvement in kidney parameters apart from higher mRNA and protein levels of Klotho. (Bikbov et al., 2020). Further, they proved that TSA upregulates Klotho at gene transcription level by transfecting HK-2 cells with mouse Klotho promoter in luciferase reporter assay. Since the renoprotective effect of TSA was significantly diminished in Klotho knockout mice shows that HDAC inhibition primarily acted via Klotho upregulation (Bikbov et al., 2020). Previous reports suggest that H3K9 modifications regulated Klotho gene in cancer cells. In a study, restoration of Klotho by inhibition of H3K9 histone methyl transferase G9a was found as one of the mechanisms behind the attenuation of kidney fibrosis (Irifuku et al., 2016). In a mouse model of kidney fibrosis, G9a expression was elevated and Klotho levels were downregulated via TGF-β1-Smad pathway. Knockdown and pharmacological inhibition of G9a by BIX01294 partially restored intrarenal Klotho levels as well as arrested kidney fibrosis. To precisely demonstrate the direct role of BIX01294 in restoring Klotho levels involved H3K9me1, the authors incubated HK-2 cells with TGF-β1. This reduced Klotho and increased H3K9Me1 expression (Irifuku et al., 2016). In a similar study, TGF-β-induced histone modifications of Klotho protein led to Klotho downregulation and kidney fibrosis (Almanza et al., 2019). Genistein treatment increased histone 3 deacetylation at Klotho's promotor region via histone acetylation along with amelioration of kidney fibrosis. Similar results were observed with TGF-β-treated HK-2 renal cells. Interestingly, Genistein could not ameliorate kidney fibrosis in Klotho knockdown mice and HK-2 cells transfected with siRNA-interfering plasmid. This indicates that genistein attenuated kidney fibrosis by restoration of Klotho levels via an epigenetically-driven mechanism (Almanza et al., 2019). Klotho is the downstream gene of the peroxisome proliferator-activated receptor gamma (PPAR-γ) and hence the post translational modification of the PPAR-γ transcriptional factor may have an important role in Klotho regulation. In the adenine mouse model of CKD, Klotho levels were first found suppressed but HDAC inhibiton using TSA restored them. While investigating the molecular mechanism behind the same the authors found that Klotho protein and mRNA levels were elevated in HK-2 cells exposed to TSA. Interestingly, the PPARγ antagonist GW9662 inhibited Klotho expression and an endogenous PPARγ ligand PGJ2 (15-deoxy-delta-12,14-prostaglandin J2) enhanced Klotho

expression and added onto the TSA driven upregulation of Klotho levels. Furthermore, ChiP assay studies revealed that exposure to TSA enhanced PPARγ binding to peroxisome proliferator responsive element on Klotho promoter (Dalton et al., 2017). Above studies provide evidence that post translational histone modifications could prevent kidney diseases via regulation of Klotho protein.

2.5.1.2 DNA hypermethylation and Klotho regulation

The process of DNA methylation involves the DNA methyltransferase enzymes (DNMTs) such as DNMT1, DNMT3a and DNMT3b, which regulate gene replication under healthy physiological conditions (Larkin et al., 2018). Hypermethylation of the DNA alters gene expression and subsequent protein translation. One of the prominent reasons for the epigenetic inheritance is DNA methylation, where the DNMTs carry methyl group from S-adenosyl methionine (SAM-CH3) to fifth carbon of the cytosine or adenine DNA nucleotides and represses gene transcription (Jing WANG, 2015; Moore et al., 2013). Interestingly, many of the *in-vivo* as well as *in-vitro* reports suggests that DNA methylation has a direct relationship with Klotho regulation in kidney disease. Jing Chen, *et al*. have reported the elevation of both renal and peripheral blood nuclear cell levels of Klotho promoter methylation in CKD patients (Chen et al., 2013). They inversely correlated the Klotho methylation level in the kidney with Klotho protein expression along the progressive decline of glomerular filtration rate and histological damage to kidney tissues in CKD patients, indicating a possible involvement of Klotho promoter hypermethylation in pathophysiology of CKD (Chen et al., 2013).

The overexpression of DNMT1 in mice with cyclosporine A-induced kidney fibrosis increases the CpG hypermethylation of the Klotho promotor (Aleksandar Denic, 2016). Cyclosporine A activated TGF-β downstream signaling pathways followed by an expression of inflammatory and fibrotic proteins and also abruptly increased DNMT1 activity which decreased the Klotho expression in fibrotic kidney. Treatment with curcumin inhibited the TGF-β signaling mediated overexpression of DNMT1 that finally restored the Klotho level that could ameliorate the kidney fibrosis (Aleksandar Denic, 2016). Also upon unilateral ureteral occlusion DNA methylation suppresses Klotho expression (Almanza et al., 2019; Chen et al., 2016). The phytoestrogenic isoflavone i.e. Genistein and anthraquinone derivative i.e. Rhein attenuate Klotho levels by inhibiting the overexpression of DNMT1/3a, which

relieved Klotho promoter hypermethylation and mitigated interstitial fibrosis (Almanza et al., 2019; Chen et al., 2016).

Systemic Klotho activity can also affect vascular biology. Vascular calcifications occur during the course of CKD progression and confer a risk for cardiovascular complications (Chen et al., 2013). Epigenetic downregulation of Klotho by uremic toxins like indoxyl sulfate promotes vascular calcifications via proliferation of vascular smooth muscle cells in CKD. Indoxyl sulfate is derived from dietary tryptophan by liver enzymes and excreted via kidney. Patients with CKD having poor kidney functions are unable to excrete indoxyl sulfate, also indoxyl sulfate is highly protein bound, thus its systemic level increases (Chen et al., 2016). In an *in-vitro* model of human aortic smooth muscle cells and an *in-vivo* model of nephrectomized Sprague Dawley rats indoxyl sulfate upregulated DNMT1/3a activity causing hypermethylation and downregulation of Klotho, which could be reversed by the 5-aza-2'-deoxycytidine and may stop the process of vascular calcification (Chen et al., 2016). Cadmium is a heavy metal and environmental contaminant with very long biological half-life (10-30 years) and toxic effects on human health including nephrotoxicity (Johri et al., 2010). People exposed to cadmium observed with epigenetically driven alteration in Klotho gene expression (Chen et al., 2013). The study revealed that, heavily exposed people to the cadmium found with high blood and urinary cadmium levels, which may upsurges hypermethylation of the RAAS protein like activator 1 and Klotho genes. Hypermethylation of the RAAS protein like activator-1 and Klotho genes associated with a decreased glomerular filtration rate and elevated levels of NAG which can be serve as an early biomarker of progression of CKD (Chen et al., 2013).

The inference of the studies directs that the DNA hypermethylation is directly allied with the activation of certain unsolicited signaling pathways which declines Klotho level and harms the renal tissues, thus the inhibition of DNA hyper methylation by targeting several epigenetically driven cell signaling pathways could elevate the Klotho level and attenuates kidney aberrations.

2.5.1.3 miRNA expressions and Klotho regulation

MicroRNAs (miRNAs) are an endogenous non-coding class of RNAs, which regulate gene expression via interacting with different binding sites of genes (Jing WANG, 2015; Mehi et al., 2014; Stefani and Slack, 2008). The regulation of miRNAs (miR) contributes to the

progression of kidney disease (Trionfini et al., 2015). Kidney-specific miRNAs include miR-146a, miR-192, miR-194, miR-204, miR-215, miR-216, and others (Sankrityayan et al., 2019a; Trionfini et al., 2015). miRNAs regulate Klotho expression in HEK293T cells, e.g. miR-339 and miR-556 bind to the 3' UTR site of the Klotho promotor and downregulate Klotho mRNA translation and protein release (Mehi et al., 2014).

One such report explored the role of miR-199a in regulation of Klotho in lupus nephritis both *in-vitro* and *in-vivo* (Ghemrawi et al., 2018). Initially, miR-199a was screened using a Klotho luciferase reporter assay. Further, miR-199a mimic transfection in HEK293 T cell significantly reduced the Klotho expression and miR-199a inhibition upregulated Klotho expression by almost two folds (Ghemrawi et al., 2018). This shows direct regulation of Klotho by miR-199a. In a similar study albiet a different miRNA, Morii, *et al.* investigated the role of miR-200c in the regulation of Klotho expression in kidney cells as well as human kidney specimens of IgA nephropathy patients (Morii et al., 2019). In kidney samples, immunohistochemistry revealed the Klotho expression. Further, using in-situ hybridization Klotho expression was inversely related to miR-200c expression. This was corroborated by a battery of *in-vitro* tests using HK-2 cells. miR-200c mimic and inhibitor transfection in the cells, was followed by Klotho protein and mRNA estimation and luciferase activity evaluation (Morii et al., 2019). miR-200c expression reduced the Klotho levels clearly demonstrating that miR-200c negatively regulates Klotho in kidney cells. In a recent study, miR-34a was reported to aggravate kidney fibrosis by downregulating Klotho expression in tubular epithelial cells (Jiang et al., 2019). The investigators used HK-2 cells and miR-34a knockout mice for establishing the effect of miR-34a on kidney fibrosis progression and underlying molecular mechanisms. Klotho levels were maintained in the miR-34a knockout model of UUO mice whereas wild type UUO mice had reduced Klotho levels underlining the role of miR-34a in regulating Klotho levels (Jiang et al., 2019). Mechanism behind tubular epithelial cell plasticity was evaluated by transfecting HK-2 cells by miR-34a mimics and inhibitors. Both protein and mRNA levels of Klotho declined after miR-34a mimic transfection (Jiang et al., 2019). Kang, *et al.* while working on tubular injury in DN found that miR-199b-5p led to tubular injury in part due to reduced Klotho levels. Bioinformatics analysis predicted Klotho to be a potential target of miR-199b-5p (Kang and Xu, 2016). Further using miR-199b-5p mimics and inhibitors as well as performing luciferase reporter assay it was confirmed that

miR-199b-5p suppressed Klotho levels and inhibiting the same could restore Klotho levels and consequent kidney functions.

However, since modulating miRNAs could produce several other effects therefore extensive studies are required to determine the safety and utility of miRNA mimics and inhibitors in clinical settings to restore Klotho levels and treat kidney disorders.

2.5.2 Non-epigenetic mechanisms of Klotho regulation

Apart from epigenetic mechanisms numerous non-epigenetic factors contribute to the downregulation of Klotho level in kidney disease, such as phosphate overload, vitamin D deficiency, hypoxia, over activity of angiotensin-II, uremic toxins, endoplasmic reticulum stress, ischemia-associated inflammation, hypomagnesemia, and albumin overload (Hidekazu Sugiura, 2012).

Few naturally occurring compounds or metabolites of microbes are described to modulate Klotho expression in the kidney. Indoxyl sulfate, an uremic toxin is metabolized by liver accumulates in serum and kidney tubules. It significantly reduced the kidney Klotho mRNA and protein levels via activation of NF-κB signaling and increased ROS production in rat kidney. Since it primarily accumulates in tubular cell thus HK-2 cells were used. Incubation of indoxyl sulfate with HK-2 cell significantly downregulated the Klotho mRNA and protein levels (2013). Pyrrolidine dithiocarbamate (PDTC) and isohelenin (ISO), NF-κB inhibitors, prevented the indoxyl sulfate-induced Klotho downregulation in the cells indicating the involvement of NF-κB signaling in indoxyl sulfate-induced Klotho downregulation. Ischemia-reperfusion and LPS mice model of AKI found with intra renal tubular damage, infiltration of granulocytes and macrophages along with activated TLR4/NF-κB signaling which further initiated cytokine production such as IL-6, TNF-α, cox-2, and apoptosis with decreased Klotho expression in kidney tissue (Li et al., 2019a). Neferine, a traditional Chinese medicine attenuated AKI and associated changes by upregulating renal Klotho level, inhibiting the NF-κB signaling and apoptosis (Li et al., 2019a). The calcineurin inhibitor tacrolimus activates the phosphatidylinositol 3-kinase (PI3K) and serine-threonine kinase Akt (AKT) pathway inhibiting the activity of forkhead box protein O transcription factor 3a (FoxO3a), which leads to oxidative damage and apoptosis in mice kidney with decrease in serum and kidney level of Klotho. Ginseng restored the serum and kidney level of Klotho, where Klotho may activate FoxO3a via inhibiting the PI3K/AKT pathway and ameliorates

kidney damage. similar findings are observed after the administration of recombinant Klotho in tacrolimus treated mice and HK-2 cells which supports the above hypothesis. (Sun Woo Lim1, 2019). Renoprotective role of ginsenosides by Klotho modulation was corroborated in a study by Li, *et al.* that reported abrogation of unilateral ureteral obstruction induced kidney fibrosis by ginsenosides through Klotho upregulation. However, the ginsinoside does not ameliorated the kidney fibrosis in Klotho knockdown rat indicates that ginsinoside may produce anti-fibrotic activity via Klotho restoration but the underlying molecular mechanisms leading to Klotho expression by ginsenoside remained unclear. Resveratrol, a well-known antioxidant, also has the potential to regulate Klotho expression in kidney. The *in-vivo* and *in-vitro* report revealed that resveratrol induced activation of activating transcription factor 3 (ATF3) which forms a complex with c-Jun and upraises Klotho expression in the kidney via binding to the promotor region of the Klotho gene, a process that ameliorated kidney injury (Hsu et al., 2014).

As discussed before, minerals have a decisive role in Klotho regulation. Magnesium decreases intestinal absorption of the phosphate via forming the complex with it and also stimulates urinary phosphate excretion via upregulating the NPT2A (Sakaguchi et al., 2019). whereas low magnesium and high phosphate diet in mice found with high systemic phosphate concentration results into phosphate induced kidney injury and decrease in Klotho expression. These effects were reversed upon magnesium supplementation (Sakaguchi et al., 2019). This indicates magnesium maintains the phosphate homeostasis which indirectly maintains the Klotho level. Albumin overload is also a putative mechanism behind Klotho regulation (Fernandez-Fernandez et al., 2018). As per the recent report, the *in-vivo* models of proteinuric kidney disease showed albumin induces ER stress via upregulation of ATF3 and ATF4 which binds to Klotho promotor region and downregulates Klotho mRNA and protein level (Delitsikou et al., 2019). Further, albumin exposed and ATF3 and ATF4 transfected HEK-293 and HK-2 cells showed reduced Klotho expression. Whereas albumin deficient mice and cells observed with decline in ATF3 and ATF4 level along with Klotho restoration indicates albumin and albumin induced ER stress have the capability to suppress the Klotho expression (Delitsikou et al., 2019). Another study where, albumin directly downregulated the expression of Klotho *in-vitro* and *in-vivo.* However, Fernandez, *et al*. did not specify the molecular mechanism behind Klotho downregulation on albumin exposure (Fernandez-Fernandez et al.,

2018). The interferon regulating factor-1 is a transcription factor considered as a renal fibrotic biomarker which decreases Klotho level via binding to C/EBP-β, the promoter region of Klotho and modulation of TNF-α signaling which worsens the condition in CKD patients (Zhao et al., 2020). This is evidenced by the experiment conducted using the UUO and adriamycin nephropathy models in mice where deletion of interferon regulatory factor-1 attenuated interstitial fibrosis by restoring Klotho levels (Zhao et al., 2020). Initially, it was found that Klotho acts as a target gene for PPAR-γ and thiazolidinedione's increased Klotho mRNA and protein levels *in-vitro* (Zhang et al., 2008). This was corroborated in another study where the PPAR-γ agonist pioglitazone significantly improved aging-related kidney disease by multiple mechanisms, Klotho upregulation being one of them. The authors further discussed that there exists PPARγ binding sites in the Klotho gene where these drugs act leading to Klotho elevation (Dalton et al., 2017). Further studies should be envisaged to unravel other endogenous Klotho upregulators and determine the underlying mode of action behind those effects. Below sections will further illustrate the non-epigenetic mechanism of Klotho regulation.

2.6 Klotho and the renin-angiotensin-aldosterone system

Klotho and the components of the RAAS are intricately connected, demonstrated by mouse models of remnant kidney, Adriamycin nephropathy, and the unilateral ureteral obstruction model of hypertension (CKD) (Zhou et al., 2015). Activation of a conventional arm of the RAAS- ACE/Ang-II/AT1R causes Klotho downregulation and/or external Klotho supplementation abolishes RAAS activation as evidenced by *in-vitro* and *in-vivo* studies (Fig. 3) (Qian et al., 2018; Zhou et al., 2015). In another study where 112 adult CKD patients were followed up for 6 years, circulating Klotho levels were positively associated with estimated glomerular filtration rate (eGFR) and inversely associated with the level of aldosterone. Furthermore, as CKD progress, systemic and renal Klotho levels decline while the level of aldosterone and kidney damage were augmented (Qian et al., 2018). In type 2 diabetic patients, Klotho levels declined along with the progressive increase in albuminuria. Treatment with the AT1R blocker losartan raised circulating Klotho levels result in a decrease in urine albumin/creatinine ratio (Lim et al., 2014). Henceforth, these reports suggest that RAAS modulation has a close association with Klotho or vice versa. Also, Klotho may exert its reno-protective effects via targeted inhibition of RAAS components and further downstream events

responsible for the progression of kidney diseases. However, these reno-protective effects of Klotho are directly or indirectly associated with RAAS is need to be explored further.

2.7 Endoplasmic reticulum stress and its effect on Klotho regulation

ER stress is a cellular stress response mainly characterized by the accumulation of misfolded or unfolded proteins in the ER (Sankrityayan et al., 2019b). However, ER stress is one of the critical underlying mechanisms in the development of kidney diseases and hence its inhibition could provide a novel therapeutic target to treat kidney diseases. A pre-clinical study by Shaoqun Shu and his team clarified that ER stress induces tubulointerstitial injury, cell apoptosis, autophagy, and inflammation in the post-ischemic kidney, which was reversed upon administration of ER stress inhibitors (Shu et al., 2018). Nonetheless, the effect of ER stress and ER stress inhibitors on Klotho regulation is a matter of investigation. The concept of attenuating kidney disease by modulating Klotho is supported through an experimental study conducted by Vasiliki Delitsikou and team in kidney epithelial- HEK-293 podocytes/HK-2 tubular cell lines and in a POD-ATTAC mouse model of proteinuric kidney disease (Vasiliki Delitsikou1, 2020). A study demonstrated that Klotho- mRNA as well as protein expression depended on the ER stress components ATF3 and ATF4. Under the condition of albuminuria, ATF3 and ATF4 repressed Klotho transcription via binding to the Klotho promoter region (Fig. 2). However, the use of the ER stress inhibitor sodium phenylbutyrate restored Klotho levels along with amelioration of proteinuric kidney disease in the POD-ATTAC mouse model and HEK-293 or HK-2 cells (Vasiliki Delitsikou1, 2020). In another study, authors have used the UUO model of renal interstitial fibrosis where, after UUO surgery, an increase in the expression of ER stress-related proteins was accompanied by more in apoptosis and fibrosis along with Klotho downregulation. However, upon administration of soluble Klotho to the UUO rats, the expression of ER stress markers was reduced with the normalization of kidney fibrosis/ functions (Fig. 2). (Qi-Feng Liu, 2015). A study represented the causal link between Klotho and ER stress using human renal epithelial (HK2), A549, SH-SY5Y neuroblastoma and HEK293 cell lines (Srijita Banerjee, 2013). The authors induced ER stress in these cells using tunicamycin and/or thapsigargin.

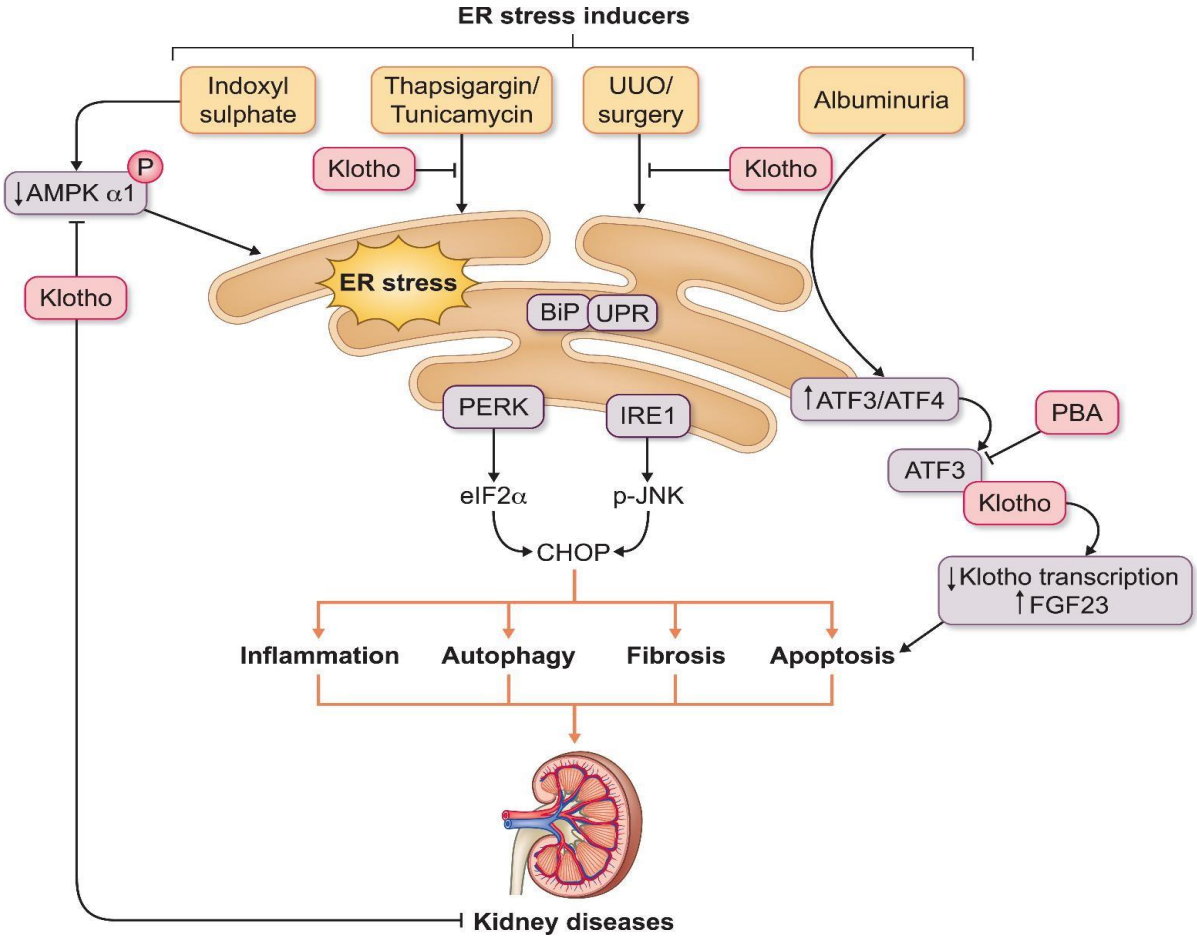


Figure 2: Molecular mechanisms of ER stress and Klotho regulation in kidney diseases.

Tunicamycin-treated cells showed high expression of unfolded protein response (UPR) signalling components such as IRE1, C/EBP homologous protein (CHOP), binding immunoglobulin protein (Bip), (PKR)-like endoplasmic reticulum kinase (PERK), p Jun N-terminal kinase (pJNK) proteins, however, cells overexpressing a mouse Klotho plasmid inhibited the expression of these ER stress-related proteins (Fig. 2). In addition, Klotho-siRNA transfection denoted elevated expression of ER stress-related and apoptotic proteins (Srijita Banerjee, 2013). Similar results were observed in human vein umbilical endothelial cells (HUVECs). Indoxyl sulfate-induced ER stress, apoptosis and cell injury in HUVECs (which mimics CKD internal environment) were attenuated upon Klotho treatment via promotion of AMP-activated protein kinase (AMPK) α1 phosphorylation (Fig. 2) (Xing, 2021). These results demonstrate that an exogenous Klotho administration or its endogenous restoration controls the ER stress as well as to attenuate the progression of kidney disease.

This ascertains that Klotho protein may regulate the ER stress or vice versa, which can be used as a therapeutic strategy for kidney diseases in future.

2.8 Crosstalk between RAAS and ER stress and their effect on Klotho regulation in kidney disease

As mentioned above, RAAS, as well as ER stress, are the key pathomechanisms involved in the progression of kidney disease. However, few studies reported that RAAS activation also modulates ER stress and vice versa (Fig. 3) (Chunling Li, 2016). Experimental DN in rats induced by Streptozotocin-triggered type 1 diabetes generated the expression of ER stress-related proteins GRP78/Bip, peIF2α, p-PERK and apoptotic protein- caspase 12 (Sun et al., 2009). The ACE inhibitor (perindopril) downregulated the expression of these proteins along with the attenuation of tubular injury and apoptosis (Sun et al., 2009). This finding suggests that RAAS activation triggers ER stress and apoptosis, which can be restored upon RAAS blockade. In support of this, Ang II-treated human kidney proximal tubular epithelial cells (PTEC) show activation of the NLRP3 inflammasome and other pro-inflammatory cytokines prompting ER stress (Jing WANG, 2015).

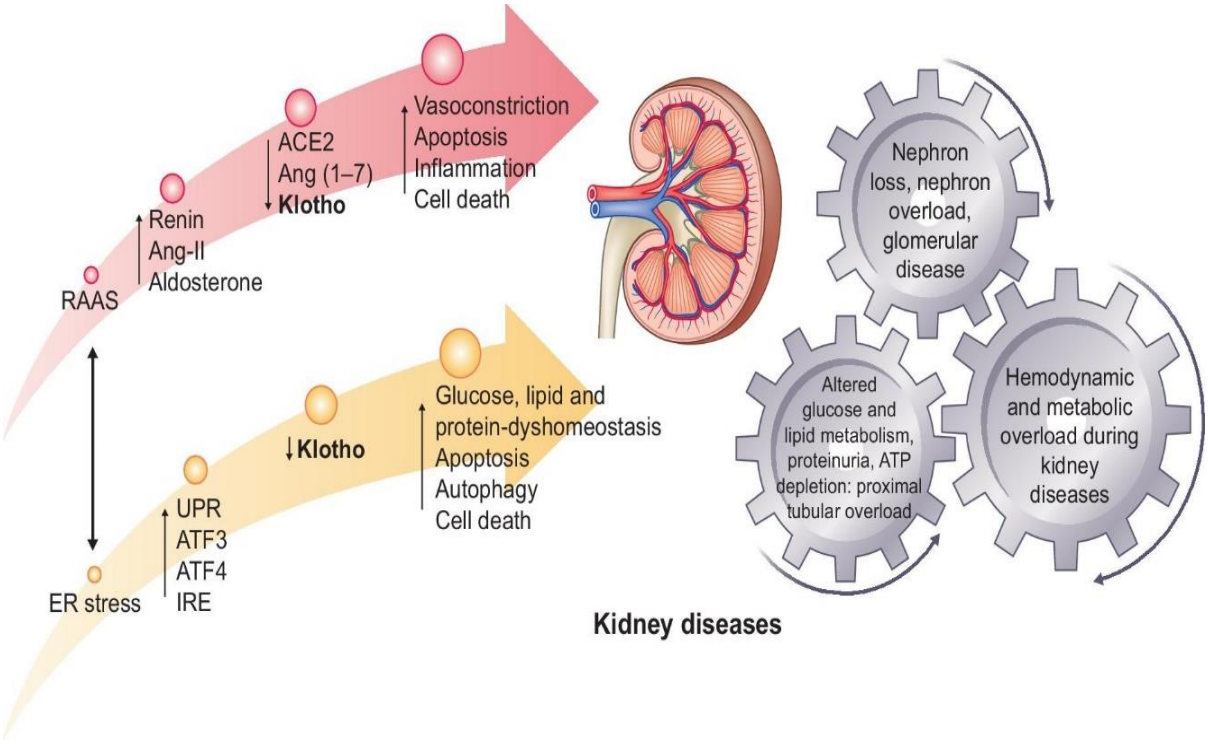


Figure 3: Crosstalk between the RAAS and ER stress in Klotho regulation: haemodynamics and metabolic overload during kidney diseases.

The ARB- telmisartan reversed ER stress further supporting that RAAS activation triggers ER stress and downstream signalling events involved in the progression of kidney disease. Thus, ACE inhibitors or ARBs may attenuate the kidney disease or its progression via inhibition of the ER stress (Chunling Li, 2016; Murugan et al., 2015). Vice versa, inhibition of ER stress impedes the RAAS-related events (Yu et al., 2020). For example, ang-II prompted podocyte injury and apoptosis via ER stress-related signalling pathways. Conversely, curcumin treatment alleviated the ang-II effects by inhibiting the ER stress (Yu et al., 2020). Also, in mice with interstitial fibrosis, aldosterone infusion triggered, ROS production as well as an expression of fibrotic proteins such as Nlrp3, IL-1β, IL-18, fibronectin, and collagen (Guo et al., 2016). Tauroursodeoxycholic acid, an ER stress inhibitor, successfully attenuated aldosterone-induced tubular injury/fibrosis via inhibiting the production of fibrotic, inflammatory proteins and reactive oxygen species (ROS) (Guo et al., 2016).

Thus, RAAS and ER stress signalling might work in conjunction with kidney disease and hence targeting one of the pathological signalling using either RAAS inhibitor or ER stress inhibitor could stop the kidney disease progression effectively. As discussed earlier, the underlying mechanism for their therapeutic action could be Klotho regulation. Moreover, future studies are required to prove this hypothesis which may provide an innovative treatment option for kidney disease.

2.9 Hemodynamic and metabolic overload in chronic kidney disease

The kidney is a vital organ as it maintains body homeostasis (Letao Fan, 2020). The nephrons of the kidney eliminate metabolic wastes and maintain an overall healthy balance of fluids, minerals and salts (Carla Viegas1, 2019; Letao Fan, 2020). Nephron loss imposes hemodynamic and metabolic overload to the remaining nephrons, which beyond certain thresholds promotes epithelial cell loss and the progression of kidney disease (Hadi Fattah, 2019). Increased workload to the remaining nephrons promotes hypoxic stress, ROS production, inflammation, and interstitial fibrosis (Fig. 3) (Schnaper, 2014). Thus, targeting hemodynamic and metabolic overload of the remaining nephrons should attenuate CKD progression. Glomerular hypertension and glomerular hypertrophy are hallmarks especially in diabetes and obesity (Avry Chagnac a, 2019; Volker Vallon 1, 2020). Single nephron hyperfiltration imposes mechanical stress on the filtration barrier (Avry Chagnac a, 2019;

Lennart Tonneijck, 2017). In addition, single nephron hyperfiltration implies an increase in reabsorption of glucose and sodium, via sodium-glucose co-transporters (SGLT1 and SGLT2), along with an increase in passive reabsorption of chloride and water in the respective proximal tubules. Additionally, SGLTs trigger the production of nitric oxide at the *macula densa*, which again further increases single nephron GFR (SNGFR) (Avry Chagnac a, 2019). In a clinical study of 1388 living kidney donors with or without diabetes, an SNGFR was studied. SNGFR is associated with risk factors for CKD such as obesity and family history of ESKD. Larger nephrons on biopsy correlated with more, glomerulosclerosis and arteriosclerosis (Aleksandar Denic, 2017). A cross-sectional clinical study also revealed that nephron hypertrophy (larger glomeruli and tubules) and nephrosclerosis (arteriosclerosis, glomerulosclerosis and interstitial fibrosis) are two interconnected processes altering kidney hemodynamics. Nephron hypertrophy indicates albuminuria, high SNGFR and larger cortical volume however, nephrosclerosis indicates lower GFR, hypertension, and lesser cortical volume (Aleksandar Denic, 2016). PTEC are key machinery involves in kidney metabolism. Increased albumin overload, high salt intake, and glucotoxicity consequences PTEC cytotoxicity, which further causes glomerular damage and kidney dysfunctions (Kimberly R. Long, 2020). Megalin and cubilin receptors are abundantly expressed by PTEC, where albumin binds and triggers the ROS production that further activates downstream inflammatory cascade and exacerbates kidney disease (Kimberly R. Long, 2020). Interestingly, attenuation of protein overload in PTEC by SGLT2 inhibition decrease the intracellular glucose and megalin O-GlcNAcylation and restored the mitochondrial function in DN (Hitomi Otomo, 2020). PTEC are high energy demanding cells where mitochondrial fatty oxidation is the source of energy, therefore mitochondrial overload could further attribute to tubular injury and thus, CKD. To prove this, Claudia Kruger and her team developed the proximal tubule- carnitine acetyltransferase (CrAT) knockout mice model of mitochondrial overload (Claudia Kruger, 2019). Study observation clarified that CrAT deletion cause impairment in fatty acid, amino acid and carbohydrate (glucose) metabolism that leads to mitochondrial dysfunction evidence by an increase in ROS production, the elevation of apoptosis, and fibrosis eventually results in tubular damage and secondary glomerulosclerosis (Claudia Kruger, 2019). Moreover, a high-fat diet in CrAT knockout mice, exacerbate the CKD rigorously, indicates impaired glucose metabolism. However, restoration

of the metabolic abnormality shows improvement in associated metabolic disorders. For example, in human kidney samples as well as *in-vitro* and *in-vivo* models of DKD, glucose-induced HIF-1α activation, and consequently HIF-1α mediated metabolic switch, tubulointerstitial damages- like macrophage infiltration and fibrosis was attenuated after the administration of the SGLT2 inhibitor dapagliflozin (Ting Cai1, 2020). Therefore, altered hemodynamics and increased metabolic overload are major contributors to the progression of CKD (Fig. 3). Importantly, restoration of altered hemodynamics and metabolic overload during kidney diseases can attenuate CKD, e.g., using SGLT2 inhibitors. Thus, targeting these abnormalities could be a novel and effective strategy for treating chronic kidney diseases.

2.10 RAAS and hemodynamic overload during kidney disease

As research is progressing, the complex functioning and novel role of RAAS in abnormal pathological remodelling evolves (dward D. Siew1, 2021). The RAAS is a key endocrine system with a substantial contribution in maintaining renal hemodynamics via controlling hypertension, hyperuricemia, albumin overload, ischemia and arteriolopathy (Fogo, 2007; laura g. Sa´ nchez-lozada, 2003). The intra-renal RAAS works independently, where inappropriate activation subsidizes hemodynamic deficiency and adverse renal outcomes (Nguyen et al., 2019). Especially, Ang-II levels are regulated by renin and angiotensinogen, which act through AT1R to promote vasoconstriction and increase sodium reabsorption. This process involves a further downstream inflammatory signalling cascade that eventually causes hypertension and kidney injury (Nishiyama and Kobori, 2018). However, administration of ACEi, ARBs and/or aldosterone antagonism shows a reversal of effects of high salt intake, proteinuria/microalbuminuria, or inappropriate activation of RAAS. Henceforth, clinically RAAS inhibitors are the first-line therapy for multiple kidney diseases (Fogo, 2007; Zhang et al., 2020b).

2.11 Endoplasmic reticulum stress in metabolic overload during kidney disease

Apart from protein synthesis and processing, ER also plays a substantial role in other cellular and metabolic processes. Likewise, metabolic overload induces ER stress and thus promotes proximal tubular injury (Fougeray et al., 2011). The high influx of glucose in tubular cells activates an unfolded protein response that leads to ER stress, followed by oxidative stress, inflammation, and apoptosis fueling kidney disease (Zhang et al., 2020a). For example, in human renal PTEC and the db/db mice model of DN, increased glucose influx into the kidney

cells, induces apoptosis and ER stress, evidenced by upregulation of the elf2α-ATF4-CHOP pathway (Shibusawa et al., 2019). However, treatment with the SGLT2 inhibitor dapagliflozin attenuated DN through glucose depletion and inhibition of the ER stress and apoptosis via deactivation of the elf2α-ATF4-CHOP pathway (Shibusawa et al., 2019). Protein dyshomeostasis, such as albumin overload/proteinuria also prompts ER stress in PTEC of the kidney. Renal biopsies of CKD patients having proteinuria as well as *in-vivo* and *in-vitro* model of proteinuric kidney disease demonstrated that albumin overload generated unfolded protein response-ER stress through an increase in cytosolic calcium, which further caused tubular apoptosis through Lipocalin 2 modulation via ATF4 in the kidney (Khalil El Karoui1, 2015). Interestingly, the above events were abolished upon treatment with the ER stress inhibitor 4-phenyl butyric acid *in-vitro* as well as *in-vivo*. Apart from altered glucose and protein metabolism, ER stress causes dysregulation of lipid metabolism which attributes to proximal tubular injury. In C57BL/6J mice and human PTEC, tunicamycin (Tm) were injected to induce ER stress (Šárka Lhoták, 2012). Tm administration leads to ER stress, sterol regulatory element-binding protein-2 (SREBP-2) activation, and lipid/free cholesterol accumulation that eventually causes apoptosis in PTEC (Šárka Lhoták, 2012). However, treatment with site-1-serine protease inhibitor (SREBP-2 deactivator), reversed the above condition.

2.12 Acute kidney injury and sodium-glucose cotransporter-2 (SGLT2) inhibitors

The incidence of severe AKI under SGLT2 therapy remains a concern. More than 100 cases of severe AKI were reported in the context of SGLT2i therapy between the year of 2013 to 2015, as per the FDA's drug safety communication report (USFDA, 2016). The extent of AKI was noted from hospitalization of the patients to the death of the patients. A study conducted by Amichai Perlman et al., evaluated the FDA adverse event report system database comprising 18,915 SGLT2i users reports between the January 2013 to the September 2016 (Perlman et al., 2017). Out of 18,915 reports, 1,224 (6.4%) were associated with an episode of AKI and 16 cases ended with death. The proportion of AKI events was higher with canagliflozin (7.3%) followed by dapagliflozin (4.8%) and empagliflozin (4.7%) (Perlman et al., 2017). Recently, Gautam Phadke and colleagues reported a case of a 66-year-old man that was treated with canagliflozin for 5 days and experienced osmotic nephropathy and AKI, manifested by hematuria, non-nephrotic-range proteinuria and elevated serum creatinine level

(Phadke et al., 2020). Patients recovered from the AKI after discontinuing treatment and dialysis, guaranteed that the AKI was persuaded by canagliflozin only. In other case study, empagliflozin seemed to cause acute interstitial nephritis in a 63-year old women and canagliflozin seemed to increase the levels of potassium and creatinine along with tubular necrosis, which in a 72-year old patient (Hassani-Ardakania et al., 2019; Rebecca Ryan 1, 2020). This notion is further supported by several reports where the incidence of AKI occurred upon SGLT2i administration (Darawshi et al., 2020; Hahn et al., 2016; Szalat et al., 2018). Apart from these, the FDA also revised the label for SGLT2i use indicating the risk of ketoacidosis and urinary tract infection (US-FDA, 2015). This concern was in conflict regarding the consideration of SGLT2i as a standard care therapy and thus restraining the use of these agents for diabetes as well as cardio-renal diseases.

2.13 Protective effects of SGLT2i in acute kidney injury and other kidney disease

After the FDA announcement and plentiful reports who described SGLT2i as an accountable cause of AKI, multiple studies were carried out to further investigate and verify the underlying mechanism and SGLT2i-induced episodes of AKI in patients with diabetes. Numerous studies revealed that the percentage of AKI episodes in patients with diabetes was significantly lower compared to other antidiabetic agents such as DPP4 inhibitors and placebo (Pasternak et al., 2020; Sridhar et al., 2020). In the post-hoc analysis study, 33 patients having type 2 diabetes and an albumin:creatinine ratio ≥100 mg/g and <3500 mg/g (11.3 – 395.5 mg/mmol) and an eGFR ≥ 45 ml/min/1.73m^2 were enrolled (Dekkers et al., 2018). Later the patients were administered with two consecutive treatments of dapagliflozin (10 mg per day) for 6 weeks with a washout period of 4 weeks in between the two treatments periods. The study revealed that dapagliflozin effectively reduced albuminuria, the levels of inflammatory proteins kidney injury molecule-1, monocyte chemotactic protein and interleukins and attenuated glomerular and tubular injury in the same patients (Dekkers et al., 2018). In addition, based on a post hoc analysis of randomized clinical trials (EMPA-REG-OUTCOME and DECLARE TIMI 58), Chang Chu et al., recommended empagliflozin as a novel option to prevent episodes of AKI in diabetic patients (Chu et al., 2019). In continuation to this, a systemic review and meta-analysis registered as PROSPERO by Brendon L Neuen and team, evaluated 38,723 participants of recent clinical studies through MEDLINE and Embase from database inception to June, 2019 (Neuen et al., 2019). This analysis confirmed that, SGLT2i significantly reduce

the risk of dialysis, transplantation as well as morbidity and mortality rate of AKI and ESKD in patients with diabetes (Neuen et al., 2019). Additionally, SGLT2i restored kidney functions via attenuation of the decreased glomerular filtration rate and urine albumin to creatinine ratio in the same patients. Henceforth, SGLT2i does not seem frequently cause AKI and additionally has the potential for considering it as a novel and clinically most effective approach for the treatment of diabetic patients at risk for kidney diseases.

2.14 SGLT2 inhibitors- Klotho axis in diabetes and cardio-renal comorbidities

Currently available data suggest that Klotho is an emerging biomarker as well as therapeutic target for the cardiovascular and kidney diseases (Lu and Hu, 2017; Ludmila Milovanova1, 2019; Neyra et al., 2020). Klotho has pleiotropic activities and therefore, has the potential to regulate multiple signaling cascade which plays the imperative role in pathophysiology of the cardio-renal diseases. Remarkably, an exogenous Klotho gene delivery and/or restoration of Klotho exhibit the tempering of diabetes and non-diabetes associated cardio-renal comorbidities (Jing Lv1, 2020; Kang and Xu, 2016). AKI is acknowledged as the state of Klotho deficiency and restoration of Klotho can lead to recovery from the AKI under conditions such as diabetes and cardiac surgery (Hu et al., 2020). Favorably, SGLT2i also has an imperative role in Klotho regulation (Sarafidis et al., 2019). This might be the reason for their protective action in AKI and diabetes associated cardio-renal comorbidities and deserves to be investigated in more detail. Additionally, there is evidence of a FGF21-Klotho axis and SGLT2 in renal glucose homeostasis, which is not explored enough yet (Li et al., 2018). Juan Navarro-Gonzalez and team, described the effect of empagliflozin, dapagliflozin and canagliflozin on Klotho expression and attenuation of type 2 diabetes and the associated CKD. In twenty-four patients receiving these SGLT2i the reduction of albuminuria and urinary TNF-α was associated with a concomitant elevation of urinary and serum Klotho levels. In addition, cultured renal tubular cells showed a dose-dependent increase in Klotho-mRNA expression upon exposure to dapagliflozin. This indicates that, SGLT2i preserve the level of Klotho as a potential mechanism for reno-protection in type 2 diabetes. This notion is further supported by the scientific statement released by American Heart Association (AHA), depicting that, SGLT2i avert downregulation of the cardioprotective factor Klotho via prevention of glucose entry into kidney cells and arresting glucotoxicity and inflammation (Rangaswami et al., 2020). In one more preclinical study, Noha A.T. Abbas et al., explored

the reno-protective effect of SGLT2i in UUO rat model (Abbas et al., 2018). Molecular and histopathological studies revealed that, UUO decreased renal mRNA levels of Klotho and increased the amount of inflammatory and fibrotic proteins such as, Nuclear factor kappa B, transforming growth factor beta, toll like receptor-4, α-smooth muscle actin, and fibronectin in kidney homogenates.

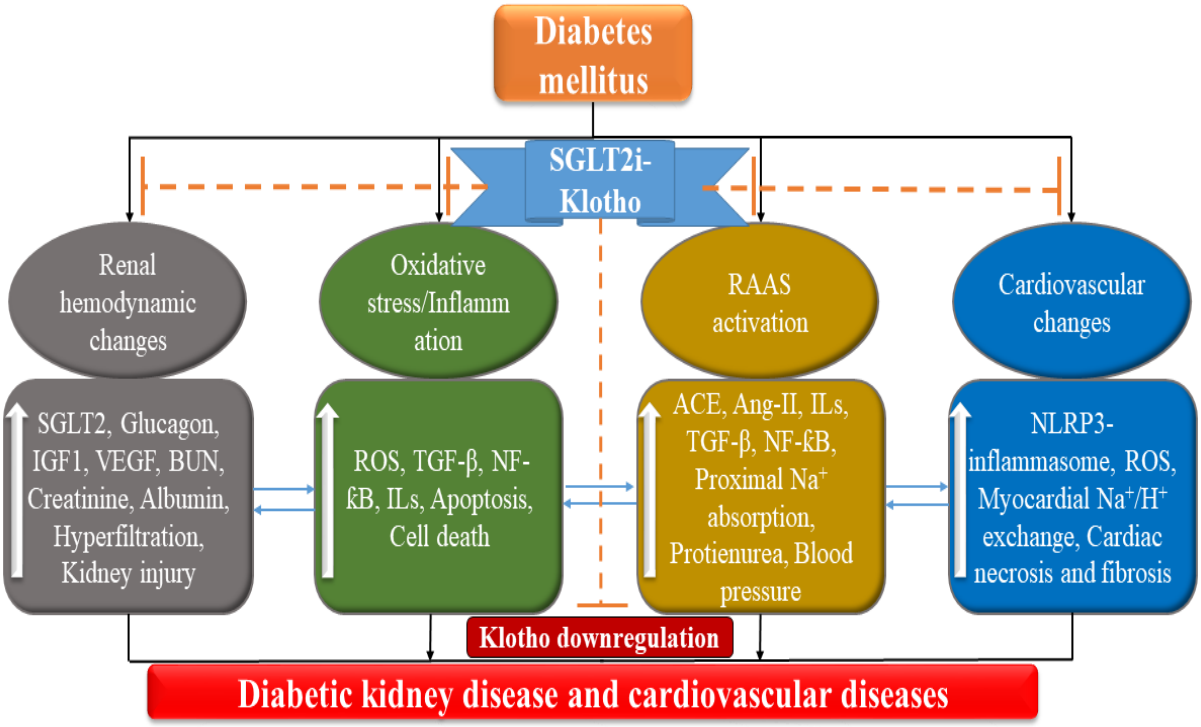


Figure 4: Conventional pathophysiology of diabetes and associated cardiorenal diseases and the effect of SGLT2is and Klotho protein on their progression.

The prophylactic and curative treatment with empagliflozin restored Klotho levels and declined the level of aforementioned proteins along with amelioration of kidney fibrosis (Abbas et al., 2018). Hence, these fundamental findings suggest that, SGLT2i regulates Klotho levels while ameliorating diabetes and its associated cardio-renal complications. The pathophysiology of diabetes-associated cardio-renal comorbidities and the putative role of the SGLT2i-Klotho axis in the attenuation of these diseases is summarized in Fig. 4.

2.15 Relationship between SGLT2i and Klotho regulation in diabetes

In March 2013, the FDA approved the new anti-hyperglycemic drugs canagliflozin, dapagliflozin and empagliflozin from the class of SGLT2 inhibitors that inhibit sodium and glucose reabsorption in the proximal convoluted tubules of the kidney (Asrih and Gariani,

2020). Physiologically, SGLT proteins recover almost 90% of the filtered glucose and hence limit glucose losses via the urine (glycosuria) (Asrih and Gariani, 2020). Hyperglycemia induces the upregulation of these proteins which, by minimizing glucose losses via the kidney, augments hyperglycemia. However, the anti-glycemic effects of SGLT2i turned out to be minor, while unexpected strong cardio-protective and nephroprotective effects now qualify SGLT2i as a potential standard treatment option for patients with type 2 diabetes. The various clinical trials such as empagliflozin cardiovascular outcome event trial in type 2 diabetic patients-removing excess glucose (EMPA-REG OUTCOME), Canagliflozin cardiovascular assessment study (CANVAS) program, Dapagliflozin on cardiovascular events (DECLARE-TIMI 58), and Canagliflozin and Renal Events in with established Nephropathy Clinical Evaluation (CREDENCE trial) demonstrated that these SGLT2i have potent cardioprotective and nephroprotective effects (McGuire et al., 2020). SGLT2i also have other beneficial effects in diabetic patients such as weight loss, regulation of blood pressure (diuresis, volume depletion and RAAS inhibitory effects), lipid metabolism and cardio-renal protection (Daniel S Hsia, 2017). While producing these effects, a correlation of SGLT2i therapy with Klotho regulation is expected.

Klotho is an anti-aging protein expressed by the multiple organs including brain, pancreas, liver, heart, and within the kidney majorly expressed by the proximal convoluted tubules. Furthermore, Klotho possesses pleiotropic properties such as regulation of mineral metabolism, modulation of the renin-angiotensin system and insulin signaling, and also owns anti-inflammatory and antioxidant properties (Dalton et al., 2017). Of note, Klotho is downregulated in diabetic patients, suggesting a link between Klotho and the progression of diabetes (Nie et al., 2017). A novel study found Klotho to be an important element in glucose and lipid metabolism under diabetic conditions (Gu et al., 2020). In a wild type and transgenic mouse model of diabetes, Klotho repressed PI3K/AKT/mTORC1 signaling and upregulated the expression of peroxisome proliferator-activated receptor-α (PPAR-α) via the insulin-like growth factor receptor (IGF1R). Molecular studies revealed that, Klotho decreased insulin resistance and improved glucose lipid metabolism via the PI3K/AKT/mTORC1-PPAR-α/IGF1R axis in mice (Gu et al., 2020). In the future, it would be interesting to explore the link between SGLT2i and Klotho regulation, and their effects on the progression of diabetes.

2.16 Relationship between SGLT2i and Klotho in cardiovascular disease

Apart from glycemic control, SGLT2i also affect blood pressure, obesity, and hence cardiovascular events in the diabetic and non-diabetic patients. Several probable mechanisms have been described for SGLT2i such as antioxidant and anti-inflammatory effects, early natriuresis, improved vascular resistance, electrolyte balance, and a decrease in plasma volume reducing the risk of heart failure (Cowie and Fisher, 2020). The Comparative Effectiveness of Cardiovascular Outcomes in New Users of SGLT2 Inhibitors (CVD-REAL) Nordic observational study reported the effect of the SGLT2i- dapagliflozin, empagliflozin and canagliflozin in patients with type 2 diabetes having the high cardiovascular risk profile (Birkeland et al., 2017). SGLT2i use was associated with less cardiovascular disease and mortality compared to other glucose-lowering drugs (Birkeland et al., 2017). Even in the absence of diabetes, SGLT2i has a protective potential on cardiovascular disease (EMPEROR- reduced trail, NCT03057977). Outcomes from this double-blind trial proved that empagliflozin can significantly reduce the rate of hospitalization and cardiovascular death in 3730 heart failure patients when compared to the placebo (Packer et al., 2020). These findings were consistent with other clinical trial programs such as Dapagliflozin on cardiovascular events (DECLARE-TIMI 58), Empagliflozin cardiovascular outcome event trial in type2 diabetic patients-removing excess glucose (EMPA-REG OUTCOME), Canagliflozin cardiovascular assessment study (CANVAS) program (Kluger et al., 2019). Apparently SGLT2i reduced the morbidity and mortality rate of cardiovascular diseases by several mechanisms. These include improvement in ventricular preload, cardiac metabolism and bioenergetics, myocardial Na^+/H^+ exchange inhibition, reduction in cardiac necrosis and fibrosis, and reduction in cytokine productions where the underline molecular mechanisms is still elusive (Verma and McMurray, 2018). SGLT2i may involve Klotho to produce these effects. Klotho is a novel cardio-protective factor and its deficiency is one of the hallmarks in the progression of cardiovascular diseases under diabetic and non-diabetic conditions. Evolving evidence supports a role for Klotho in the development of diabetes-related cardio-vascular diseases (Guo et al., 2018). Klotho attenuated hyperglycemia-induced cardiac injury or diabetic cardiomyopathy via inhibition of inflammation, mitochondrial dysfunction, reactive oxygen species production, and apoptosis in the diabetic mice (Guo et al., 2018). These outcomes were further supported by *in-vitro* studies.

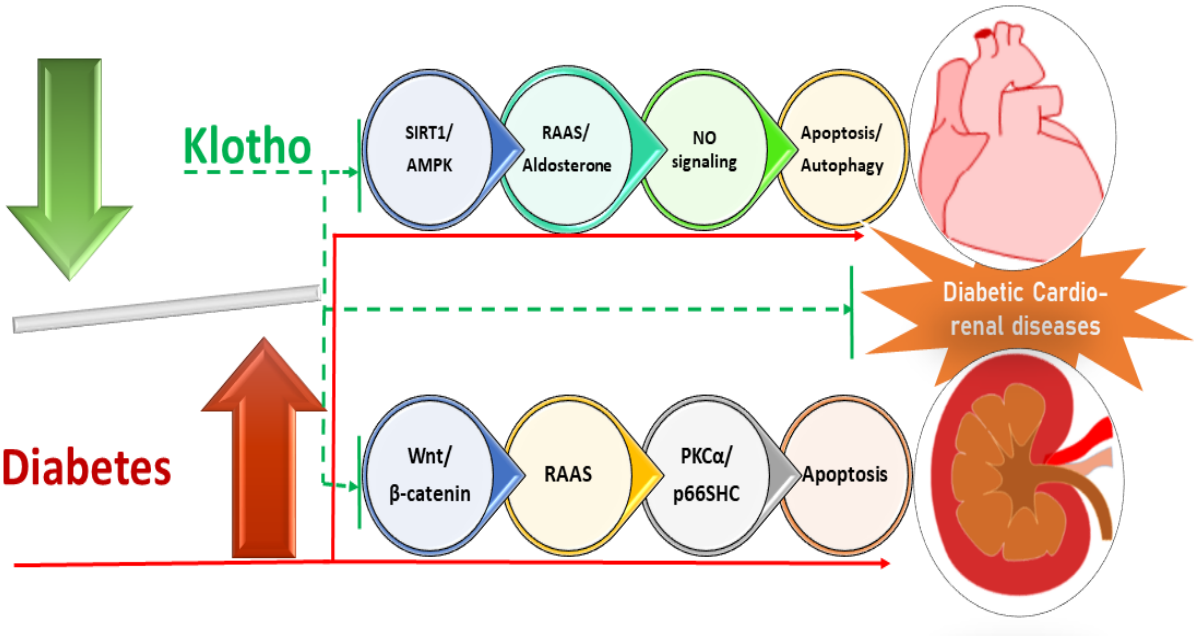


Figure 5: Klotho and its prevention of diabetic kidney and cardiovascular events.

In addition, Klotho treatment attenuated streptozotocin-induced diabetic cardiomyopathy via inhibition of the NLRP3 inflammasome pathway and apoptosis (Li et al., 2019b). Preclinical data strongly suggest that, decline in Klotho levels due to kidney dysfunction noticeably contributes to the incidence of cardiac events in patients with CKD. Of note Klotho restoring, significantly reduces the rate of CVD events in CKD patients (Bi et al., 2020; Memmos et al., 2019). Klotho ameliorates these conditions via modulation of several key pathways including, SIRT1 and AMPK signaling, NO signaling, RAAS inhibition, decrease in aldosterone level, stimulation of autophagy, inhibition of apoptosis and ROS generation (Aroor et al., 2018; Neyra et al., 2020; Olejnik et al., 2018). Modulation of these signaling pathways by Klotho eventually averts cardiovascular events such as, cardiac remodeling, cardiac hypertrophy, cardiomyopathy and cardiac dysfunction (Fig. 5).

2.17 Relationship between SGLT2i and Klotho in diabetic kidney diseases

Diabetic kidney diseases (DKD) is a common late complication of diabetes and, if left untreated, may lead end stage kidney disease and death (Anders et al., 2018). SGLT2i offer enormous benefits for DKD, which had first been attributed to their capacity to reinstall the tubulo-glomerular feedback mechanism and reduce glomerular hyperfiltration, however the definite mechanisms remain mysterious (Anders et al., 2016). As per the clinical trial outcomes in type2 diabetes, SGLT2i yields reno-protection by two novel physiological effects

which is not produced by other anti-hyperglycemic drugs: ketogenesis and erythrocytosis (Packer, 2020). Hiroshi Maegawa and colleagues, reported empagliflozin to induce ketogenesis offering reno-protection in mouse model of DKD (Tomita et al., 2020). Empagliflozin treatment raised the endogenous ketone body level (ketogenesis), which further triggered ATP depletion and inhibited the mechanistic target of rapamycin complex 1 (mTORC1). mTORC1 inhibition ensued attenuation of podocyte damage/kidney injury and also halt the increasing proteinuria in mice with DKD (Tomita et al., 2020). Secondly, SGLT2i suppresses hypoxia-inducible factor-1α (HIF-1α) and prompts HIF-2α which endorse the production of erythropoietin and blood flow to the kidney (Packer, 2020). According to a multinational observational cohort study, SGLT2i therapy slowed down the rate of kidney function decline and also lowered the risk of adverse kidney events when compared with other hypoglycemic therapies (Heerspink et al., 2020a). The CREDENCE (Canagliflozin and Renal Events in Diabetes with stablished Nephropathy Clinical Evaluation) trial, further confirmed the utility of SGLT2i in DKD first using a primary renal endpoint (Cherney et al., 2019). Recently, The Kidney Disease: Improving Global Outcomes (KDIGO) recommended the metformin and SGLT2i as first line treatment for the management of type 2 diabetes in patients with CKD (Kidney Disease: Improving Global Outcomes Diabetes Work, 2020). Several other clinical trials are ongoing to explore the role of SGLT2i in diabetes and cardio-renal disorders (table 2). Apart from SGLT2i, the novel protein- Klotho is also the topic of attention. Kidney tissue from the patients with DKD displayed low levels of Klotho mRNA and Klotho protein compared to normal kidney tissue. In parallel to this, the db/db mice model of DN found with diminished amount of Klotho originating from glomerular endothelial cell injury by persuading the inflammation and fibrosis via Wnt/β-catenin signaling and RAAS pathway.

Table 2: Clinical trials with SGLT2 inhibitors in diabetes and cardio-renal diseases.

Clinical trials title/ Identifier	**Intervention/ treatment**	**Disease**	**Study objective/ purpose**	**Status of the study**
SGLT2 Inhibitor Adjunctive Therapy to Closed Loop Control in Type 1 Diabetes	Empagliflozin IQ insulin pump	Type 1 diabetes	The aim of the study is to assess efficacy and safety of combining the SGLT2i with closed loop control.	Phase 1 Recruiting

Mellitus (NCT04201496)				
Sodium-glucose Cotransporter 2 (SGLT2) Inhibitors and Risk of Cardiovascular Events (NCT03939624)	SGLT2 inhibitors Dipeptidyl peptidase-4 (DPP-4) inhibitors	Type 2 Diabetes mellitus Myocardial Infarction Ischemic Stroke Cardiovascular Death Heart Failure	The purpose of the study was to evaluate the risk of major adverse cardiac events (MACE) associated with the use of SGLT2i in comparison with the use of DPP-4 inhibitors in type 2 diabetes patients.	Phase 4 Completed 419734 participants
Safety of Sodium-glucose Cotransporter 2 (SGLT2) Inhibitors Among Patients With Type 2 Diabetes (NCT04017221)	SGLT2i (ATC A10BK) Dipeptidyl peptidase-4 (DPP-4) inhibitors (ATC A10BH) Other anti-diabetic drugs (ATC A10A and A10B (excluding A10BH and A10BK))	Type 2 diabetes mellitus Urosepsis Diabetic ketoacidosis Lower extremity amputation	The main objective of the study was to compare the risk of serious adverse events associated with the use of SGLT2i to DPP-4 inhibitors among patients with type 2 diabetes. Specifically, to evaluate the risk of severe urinary tract infection, diabetic ketoacidosis and lower extremity amputation.	Phase 4 Completed 1249636. Participants
SGLT2 Inhibitor or Metformin as Standard Treatment of	Dapagliflozin Metformin	Type 2 diabetes mellitus	The purpose of the study is to compare the SGLT2 inhibitors with metformin as standard	Phase 4 Recruiting

Early-Stage Type 2 Diabetes (SMARTEST) (NCT03982381)			treatment in early type 2 diabetes. Further to address the efficacy with respect to clinically important macro- and microvascular events	4300 participants
SGLT2 Inhibitors in Glomerular Hyperfiltration (EMPATHY) (NCT04143581)	Empagliflozin	Obesity Non-diabetic CKD	The study expected that, SGLT2 inhibition therapy might reduce the sodium overload and volume expansion which, along with secondary hypertension, may further contribute to kidney hyperperfusion and glomerular hyperfiltration in obesity and CKD	Phase 2 Not recruited yet
Safety and Effectiveness of SGLT2 Inhibitors in Patients with Heart Failure and Diabetes (REFORM) (NCT02397421)	Dapagliflozin	Heart failure Diabetes	Diabetic patients are at higher risk of heart failure which may cause shortness of breath, decreased ability to exercise and premature death in some cases due to inability of the heart to pump the blood throughout the body. The objective of the study was to assess the safety and benefits of SGLT2i (Dapagliflozin), in treating heart failure and diabetes.	Phase 4 Completed 56 participants
Effects of the SGLT2 Inhibitor Empagliflozin in Patients with Euvolemic and Hypervolemic	Empagliflozin	Hyponatremia SIADH	The study aimed to evaluate the effect of empagliflozin in eu- and hypervolemic hyponatremia.	Phase 4 Not recruiting yet

Hyponatremia (EMPOWER) (NCT04447911)		Liver failure Kidney failure		
Performance Under SGLT2-Inhibitors in Humans (PUSH) (NCT03422263)	Empagliflozin Dapagliflozin Canagliflozin	Type 2 diabetes mellitus	This study was conducted to investigate the early effects of SGLT2i on the physical performance of patients with type 2 diabetes mellitus compared to patients under other therapy regimes. Patients were divided into 3 groups: I - Patients with type 2 diabetes and SGLT2i II - Patients with type 2 diabetes and without SGLT2i and III - Patients without type 2 diabetes but with similar comorbidities to the previous groups.	Phase 4 Completed 450 participants
Sodium-glucose Co Transporter 2 (SGLT2) Inhibitor and Endogenous Ketone Production (NCT03852901)	Empagliflozin	Diabetic kidney disease	To evaluate the effect of empagliflozin on ketone bodies production	Phase 1 Recruiting
Efficacy and Safety of Dapagliflozin in Acute Heart Failure (DICTATE-AHF) (NCT04298229)	Dapagliflozin Protocolized diuretic therapy	Acute heart failure Type 2 diabetes mellitus	DICTATE-AHF a randomized trial will be conducted to assess the effect of the addition of dapagliflozin to patients with diabetes hospitalized with acute decompensated heart	Phase 3 Recruiting

			failure (ADHF). Participants will be recruited following an initial standard evaluation in the ED and randomized within 24 h of presentation for ADHF in a 1:1 fashion to protocolized diuretic therapy or dapagliflozin + protocolized diuretic therapy	
Effects of SGLT2 Inhibitor on Type 2 Diabetic Patients Undergoing Cardiac Surgery (NCT04340908)	Dapagliflozin	Type 2 diabetes mellitus Cardiac Surgery	This study is planned for evaluation of impact of SGLT2i treatment of one year on cardiac function, postoperative complications and long-term cardiovascular mortality in diabetic patients undergoing cardiac surgery. The echocardiography is used to evaluate the cardiac function in diabetic patients during perioperative cardiac surgery.	Phase 4 Recruiting
Studies of Empagliflozin and Its Cardiovascular, Renal and Metabolic Effects (SUGAR-DM-HF) (NCT03485092)	Empagliflozin	Heart Failure Diabetes mellitus	The investigator expected that empagliflozin causes haemodynamic, cardiac, and renal benefits compared to placebo over 36 weeks in heart failure patients with type 2 diabetes (or pre-diabetes), leading to measurable improvements in clinical	Phase 4 Completed 105 participants

			measures of cardiac structure and function (LVESVI, and LV strain) as well as renal blood flow.	
LIRA-ADD2SGLT2i - Liraglutide Versus Placebo as add-on to SGLT2 Inhibitors (NCT02964247)	Liraglutide	Type 2 diabetes mellitus	The trial is conducted in Asia, Europe, North America and South America. The aim of the study is to compare the effect of liraglutide 1.8 mg/day versus placebo as add-on to an SGLT2 inhibitor with or without metformin on glycemic control in subjects with type 2 diabetes mellitus.	Phase 3 Completed 303 participants
SGLT2 Inhibition and Left Ventricular Mass (EMPATROPHY) (NCT02728453)	Empagliflozin Glimepride	Type 2 diabetes mellitus	Study was planned to assess left ventricular mass, function, and lipid content in patients with type 2 diabetes mellitus using cardiac magnetic resonance imaging and spectroscopy as well as echocardiography before and after empagliflozin or glimepiride treatment. They expect to observe improvements in left ventricular mass, function, and fat content with empagliflozin.	Phase 4 Terminated (insufficient numbers)
A Study of Dulaglutide (LY2189265) in Participants with Type 2 Diabetes Mellitus	Dulaglutide Placebo SGLT2 inhibitor Metformin	Type 2 diabetes mellitus	The main purpose of this study is to evaluate the efficacy and safety of the study drug known as dulaglutide when added to sodium-glucose co-transporter 2 (SGLT2)	Phase 3 Completed 424 participants

(AWARD-10) (NCT02597049)			inhibitors in participants with type 2 diabetes mellitus.	
Canagliflozin and Trial Analysis-Sulfonylurea (CANTATA-SU) SGLT2 Add-on to Metformin vs Glimepiride (NCT00968812)	Glimepride Canagliflozin Metformin	Type 2 diabetes mellitus	This study designed to demonstrate the efficacy, safety, and tolerability of canagliflozin (JNJ-28431754) compared with glimepiride in patients with type 2 diabetes mellitus with inadequate control despite treatment with metformin.	Phase 3 Completed 1452 participants
Evaluation of the Effects of Canagliflozin on Renal and Cardiovascular Outcomes in Participants with DN (CREDENCE) (NCT02065791)	Canagliflozin Placebo	Type 2 diabetes mellitus DN	The study objective was to assess the renal and vascular protective effects of canagliflozin in reduction of the progression of renal impairment compared to placebo in type 2 diabetes participants, with stage 2 and 3 CKD and microalbuminuria taking the angiotensin-converting enzyme inhibitor or angiotensin receptor blocker.	Phase 3 Completed 4401 participants
CANVAS - Canagliflozin Cardio-vascular Assessment Study (CANVAS) (NCT01032629)	Canagliflozin Placebo	Type 2 diabetes mellitus Cardiovascular diseases Risk factors	The purpose of the study is to evaluate the effect of canagliflozin in type 2 diabetes patients with regard to cardiovascular risk for major adverse cardiac events (MACE). Additionally, the evaluation of overall safety, tolerability, and	Phase 3 Completed 4330 participants

			effectiveness of canagliflozin. The data from this study will be combined with the data from CANVAS-R study (Study of the Effects of canagliflozin on Renal Endpoints in Adult Subjects with T2DM, NCT01989754).	
BI 10773 (Empagliflozin) Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients (EMPA-REG OUTCOME) (NCT01131676)	BI 10773 low dose Placebo BI 10773 high dose BI 10773 high dose Placebo BI 10773 low dose	Type 2 diabetes mellitus	The objective of the study is to investigate the safety of BI 10773 treatment in patients with type 2 diabetes mellitus and high cardiovascular risk.	Phase 3 Completed 7064 participants
Multicenter Trial to Evaluate the Effect of Dapagliflozin on the Incidence of Cardiovascular Events (DECLARE-TIMI58) (NCT01730534)	Dapagliflozin Placebo	Diabetes mellitus, non-insulin-dependent High risk for cardiovascular event	To determine the effect of dapagliflozin on cardiovascular outcomes when added to current background therapy in patients with type 2 diabetes with either established cardiovascular disease or risk factors.	Phase 3 Completed 17190 participants
Comparative Effectiveness of Cardiovascular Outcomes in New Users of SGLT2 Inhibitors (CVD-	SGLT2 inhibitors DPP-4 inhibitors	Type 2 diabetes mellitus	In patients with type 2 diabetes mellitus evaluating the comparative effectiveness of initiating treatment with a SGLT2i versus another	- Completed 99999 participants

REAL) (NCT02993614)	Glucagon-like peptide-1 receptor agonists		glucose-lowering drug. To compare the risk of all-cause mortality and clinically relevant cardiovascular outcomes respectively in patients who are new users of SGLT2i with those who are new users of other glucose-lowering drugs.	
Empagliflozin in Patients with Chronic heart Failure (EMPEROR-Reduced) (NCT03057977)	Empagliflozin Placebo	Heart failure	To investigate the safety and efficacy of empagliflozin versus placebo on top of guideline-directed medical therapy in patients with heart failure with reduced ejection fraction.	Phase 3 Completed 3730 participants

However, Klotho administration reversed this condition along with inhibition of the aforementioned signaling cascades (Wang et al., 2019). A clinical study with type 2 diabetic patients, stage 3-4 CKD and serum and urinary Klotho of 295.9 pg/mL and 54.1 ng/g, respectively, further suggest the therapeutic potential of Klotho (Navarro-Gonzalez et al., 2018). After 1-year treatment with pentoxifylline, eGFR had declined by 3.9% and albuminuria by 12.5%, along with increase in serum Klotho by 5.9% and urinary Klotho by 9.3% (Navarro-Gonzalez et al., 2018). This finding was further supported by *in-vitro* studies revealing that Klotho preservation could be involved in the protective effects of pentoxifylline. An experimental *in-vitro* and *in-vivo* study demonstrated a nephroprotective role of Klotho for the progression of DN (Wei Jianga, 2019). DN involves activation of PKCα/p66SHC pathway and downstream events such as, proteinuria, apoptosis and podocyte injury which ensued Klotho deficiency. Overexpression of Klotho *in-vivo* and *in-vitro* ameliorate the DN by inhibiting the PKCα/p66SHC pathway and downstream events (Fig. 4) (Wei Jianga, 2019). These reports are sturdily signifying the potential application of SGLT2i and significance of Klotho regulation in the DKD.

2.18 Relationship between SGLT2i and Klotho in non-diabetic kidney disease

Diabetic and/or non-diabetic patients with CKD are extremely prone to the high risk of adverse kidney and cardiovascular complications. Role of SGLT2i in CKD under diabetic condition is well explored however under non-diabetic condition their effect was unknown. Hence the DAPA-CKD trial (NCT03036150), examined the effect of dapagliflozin in 4304 CKD patients having the GFR of 25-75 mL/min/1.73m^2 and albuminuria in the range of 2000-5000 mg/g, with mean age 62 years (Heerspink et al., 2020b). The study suggests that dapagliflozin is equally effective in CKD patients, irrespective of presence or absence of diabetes. Dapagliflozin tempered the decline in eGFR by 50%, and also the end stage kidney disease or death from any other cardio-renal causes (Heerspink et al., 2020b). The primary outcome from EMPEROR trial demonstrated that among the 3730 heart failure patients those receiving 10 mg/day of empagliflozin showed a better estimated GFR compared to the placebo group (-0.55 vs. -2.28 ml/min/1.73 m^2 of body surface area/year, $p<0.001$). Additionally, the empagliflozin-treated group had a lower risk of adverse kidney outcomes evidenced by a reduction in occurrence of renal death or need of renal transplantation or dialysis, by 35 to 50% compared to the placebo group (Packer et al., 2020). As per the latest pre-clinical data, Klotho is one of the emerging, diagnostic and therapeutic target for CKD (Neyra et al., 2020). In normal physiological conditions, Klotho is abundantly found in the kidney whereas CKD is the state of Klotho deficiency. Interestingly, alteration in Klotho level shown independent association with CVD events and mortality in non-diabetic CKD patients (Yang et al., 2020). A median follow-up from 2014-2019, in patients with non-diabetic CKD (stage 2-5 predialysis), low serum levels of Klotho were associated with higher all-cause mortality (HR = 7.09; 95% CI 1.59-31.25) and also a higher cardio-vascular event risk (HR= 3.02; 95% CI 1.45-6.30) compared to patients with higher Klotho levels (Yang et al., 2020). These studies imply the importance of SGLT2i and Klotho in CKD progression and related complications. However, more studies are necessary to further explore the affiliation between SGLT2i and Klotho in CKD.

3. Background and objectives

3.1 Background

AKI is a detrimental health issue common amongst hospitalized and comorbid patients like diabetics (Abebe et al., 2021). The need for early biomarkers and potential therapeutic targets is in demand. Klotho has been in the limelight of research from the last two decades for its biomarker characteristics and reno-protective potentials (Javier A. Neyra, 2020). It is known that Klotho is downregulated during kidney disease. However, the effects of hyperglycemia on Klotho level is ambiguous. Controversial reports are available regarding hyperglycemia's effect on the Klotho level. Hence, it becomes essential to evaluate the impact of hyperglycemia on Klotho first. Also, the plasma, urine and renal level of Klotho are not at all explored in AKI under the hyperglycemic condition, and as per statistics is one of the highest occurring comorbidity of AKI.

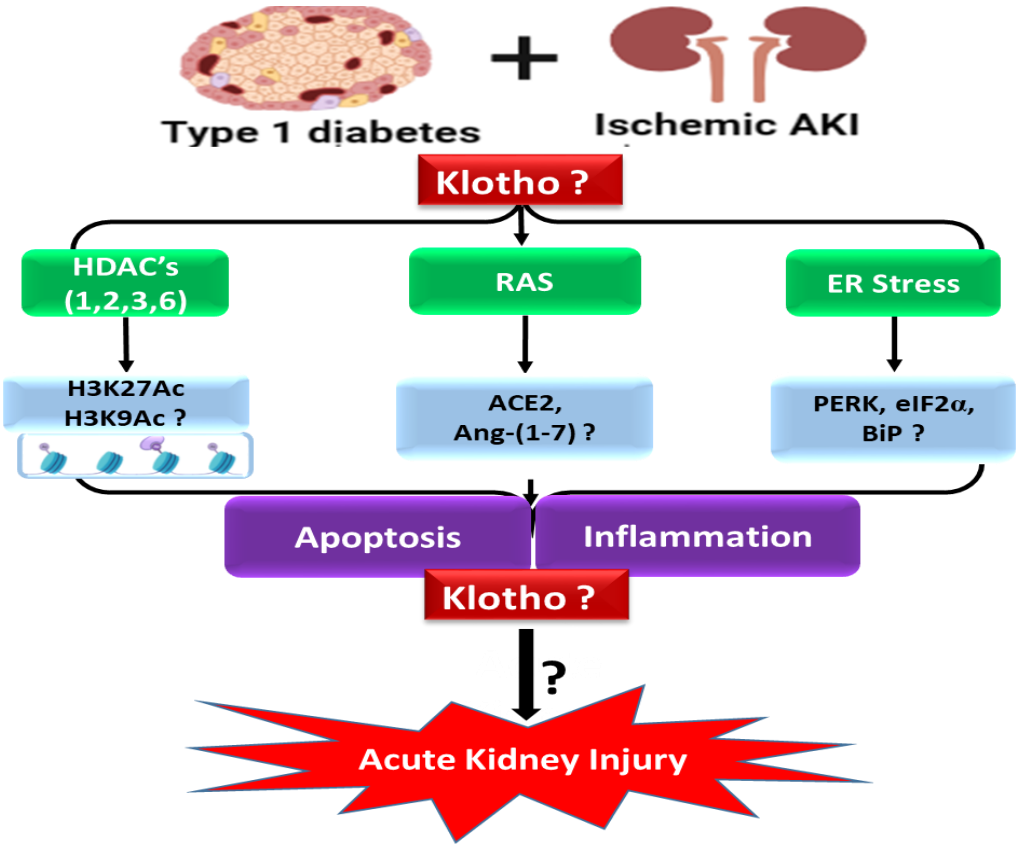


Figure 6: Hypothesis and existing gap in research against the Klotho regulation in ischemic AKI under diabetic and non-diabetic conditions.

The underlying molecular mechanisms of Klotho regulation during ischemic AKI under normal or hyperglycemic condition is unexplored (Fig. 6). Epigenetic and non-epigenetic mechanisms can be involved in Klotho regulation wherein the clear picture is not available. Epigenetic mechanisms like post-translational histone modifications have an impact on regulation of certain genes/proteins (Fontecha-Barriuso et al., 2018). However, its effects on Klotho in AKI-hyperglycemic comorbidity is inadequately known. Non-epigenetic mechanisms like renin-angiotensin system and endoplasmic reticulum stress are reported to modulate Klotho in other diseases. However, particularly the role of non-conventional RAAS role on Klotho regulation during ischemic AKI in presence or absence of diabetes is unexplored. Similarly, ER stress in known to be triggered during AKI and diabetes as well (Almanza et al., 2019). Moreover, it also has relation with Klotho regulation during chronic kidney disease. But the association between Klotho and ER stress during AKI under hyperglycemic condition is unknown. Also, the molecular signaling pathways such as apoptosis and inflammation shares the link with histone modifications, RAAS and ER stress (De Blasio et al., 2017; Fougeray et al., 2011). Thus, to address these existing gaps in research, we have proposed to study the epigenetic (kidney specific histone deacetylases) and non-epigenetic (RAAS and ER stress) mechanisms of Klotho regulation in AKI under diabetic and non-diabetic conditions (Fig. 6).

3.2 Objectives

1. To study the role of HDAC in the regulation of Klotho in the development of AKI under hyperglycemic condition.

2. To study the role of ACE2 in the regulation of Klotho in the development of AKI under hyperglycemic condition.

3. To study the role of ER stress in the regulation of Klotho in the development of AKI under hyperglycemic condition.

4. Methodology

4.1 Materials

All the instruments and materials used throughout the study are enlisted in the below.

Table 3: Instruments, make and country.

#	**Name of the instrument**	**Make**	**Country**
1.	Cell culture - Laminar air-flow safety cabinet - CO_2 incubator	Thermo Fisher	USA
2.	Microscope (Olympus- BX41) (Zeiss: Vert.A1)	Olympus Zeiss	USA Germany
3.	Laser Doppler	Moor VMF-LDF2	UK
4.	Biochemical analyzer	ERBA EM-200	Germany
5.	Microtome (Leica RM2125 RTS)	Leica Biosystems	Germany
6.	-80°C Upright Ultra-Low Temperature Freezers	Thermo Fisher	USA
7.	Mini-PROTEAN® Tetra Cell Vertical electrophoresis unit	Bio-Rad	USA
8.	Trans-Blot® SD- Semi-Dry transfer apparatus	Bio-Rad	USA
9.	Chemic Doc	Bio-Rad	USA
10.	C1000 Touch™ Thermal Cycler	Bio-Rad	USA
11.	LightCycler® 96-RT-PCR System	Roche	Germany
12.	DynaMag-2	Thermo Fisher	USA
13.	Biorad Universal Hood II Gel Doc System	Bio-Rad	USA

Table 4. Biochemical kits and chemicals.

#	**Name of the product**	**Suppliers**
1.	Streptozotocin	Sigma-Aldrich India (Delhi, India)
2.	TSA	TCI Chemicals (Tamilnadu, India)
3.	Diminazene Aceturate	Sigma-Aldrich India (Delhi, India)
4.	MLN-4760	Tocris Biosciences, (Bristol, UK)
5.	Tauroursodeoxycholic acid	Sigma-Aldrich India (Delhi, India)
6.	Tunicamycin	Sigma-Aldrich India (Delhi, India)
7.	Biochemical estimation kits for glucose, urea, creatinine	Accurex Biomedical Pvt. Ltd. (Mumbai, Maharashtra, India).
8.	ACE2, Ang (1-7), KIM-1, NGAL, Klotho ELISA kits	Elabscience (Wuhan, China)
9.	Annexin V-FITC kit	Abcam (Boston, USA)
10.	Sodium azide	Sigma-Aldrich India (Delhi, India)

4.2 Animal and cell culture studies

All the animal experimental procedures and animal care were approved by the Institutional Animal Ethics Committee (IAEC), Birla Institute of Science and Technology, (BITS) Pilani (Protocol number: IAEC/RES/27/13/REV-1/30/27). Experimental male Wistar rats (200-220 g) were procured from the Central Animal Facility (CAF), BITS Pilani. All animal studies were performed with the compliance of ARRIVE guidelines (Percie du Sert et al., 2020). Rats were provided with the standard environmental conditions, food and water ad libitum. Rat kidney tubular epithelial cells (NRK52E) were purchased from NCCS, Pune, India. Cells were cultured in low glucose (5.5mM) and/or high glucose (30mM) containing Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution. NRK52E cells were grown under the humidified condition of 5% CO_2, 95% air and 37^0C temperature.

4.2.1 Type 1 diabetes induction

Type 1 diabetes was induced by injecting a single dose of STZ (55 mg/kg, *i.p.*), dissolved in ice-cold sodium citrate buffer (0.01 m and pH 4.4) (Goru et al., 2016). NC rats with the same age group received vehicle sodium citrate buffer. After the 48 h of STZ administration, fasting blood glucose level was measured and those animals having the blood glucose level above >16 mmol/L were considered diabetic animals.

4.2.2 Induction of bilateral ischemia-reperfusion renal injury

Bilateral renal ischemic reperfusion injury (BIRI) was developed in the diabetic as well as non-diabetic rats (Sharma et al., 2019b). Briefly, after one week of diabetes induction, diabetic as well as non-diabetic rats were injected with normal saline solution (20 ml/kg s.c.) to avoid fluid loss during the surgery. Rats were anaesthetized using pentobarbital sodium at the dose of 50 mg/kg, *i.p.* After the loss of consciousness, indicated by loss of pedal pain and corneal reflexes, the half-inch incision was made on the left and right flank portion of the abdomen. Both the kidneys were pulled out using blunt forceps and renal vascular pedicles were clamped for the next 20 min. using the bulldog clamp. Clamps were released and suturing was done using absorbable and non-absorbable sutures for muscles and skin layers respectively. Then, saline was administered to the animals and animals were maintained for next 24 h for reperfusion. After the 24 h of reperfusion, all animals were sacrificed, humanly.

4.2.3 Cell culture and experimental design

To mimic the ischemic renal injury condition we used an *in-vitro* model of chemical hypoxia using 10mM sodium azide (ATP depletion or severe hypoxia) prepared in serum-free DMEM (Kurian and Pemaih, 2014). Serum starved NRK52E cells were exposed to 10mM sodium azide for 3 h followed by 2 h of incubation in complete DMEM (to mimic *in-vivo* reperfusion). To evaluate the development of hypoxia reperfusion injury, cells were checked for apoptosis using Annexin-V FITC kit via flow cytometry analysis (Kwan et al., 2016). The NRK52E cells with 70% confluency were exposed to normal glucose (5.5 mm) and high glucose (30 mm) conditions. The respective treatments were given followed by HRI and sample collection. Cells lysate was used for the experiments like western blotting, RT-PCR, FACs analysis, etc.

4.2.4 Study plans and treatment strategy

For Study 1: The objective of this study was to study the epigenetic ways to restore the Klotho expression in AKI-Diabetes comorbidity. To achieve this, IRI and HRI were developed in diabetic rats and, NRK52E cells under high glucose conditions respectively, to mimic the AKI condition (Fig. 9). Further, following groups were made:

In-vivo study:

Both diabetic and non-diabetic rats were subdivided into three groups-: a) **NC/DC**- Normal control (NC)/Diabetic control (DC) served as respective controls, b) **NC-I/R/DC-I/R**- NC rats subjected to 20 min bilateral ischemic renal injury and 24 h reperfusion/ DC rats subjected to 20 min IRI and 24 h reperfusion, c) **NC-I/R+TSA/DC-I/R+TSA**- NC-I/R with TSA (0.5 mg/kg, *i.p.*), dissolved in dimethyl sulfoxide (DMSO), and injected at 16 h and 30 min before the surgery/ DC-I/R with TSA (0.5 mg/kg, *i.p.*), and injected at 16 h and 30 min before the surgery (Fig. 7A) (Levine et al., 2015).

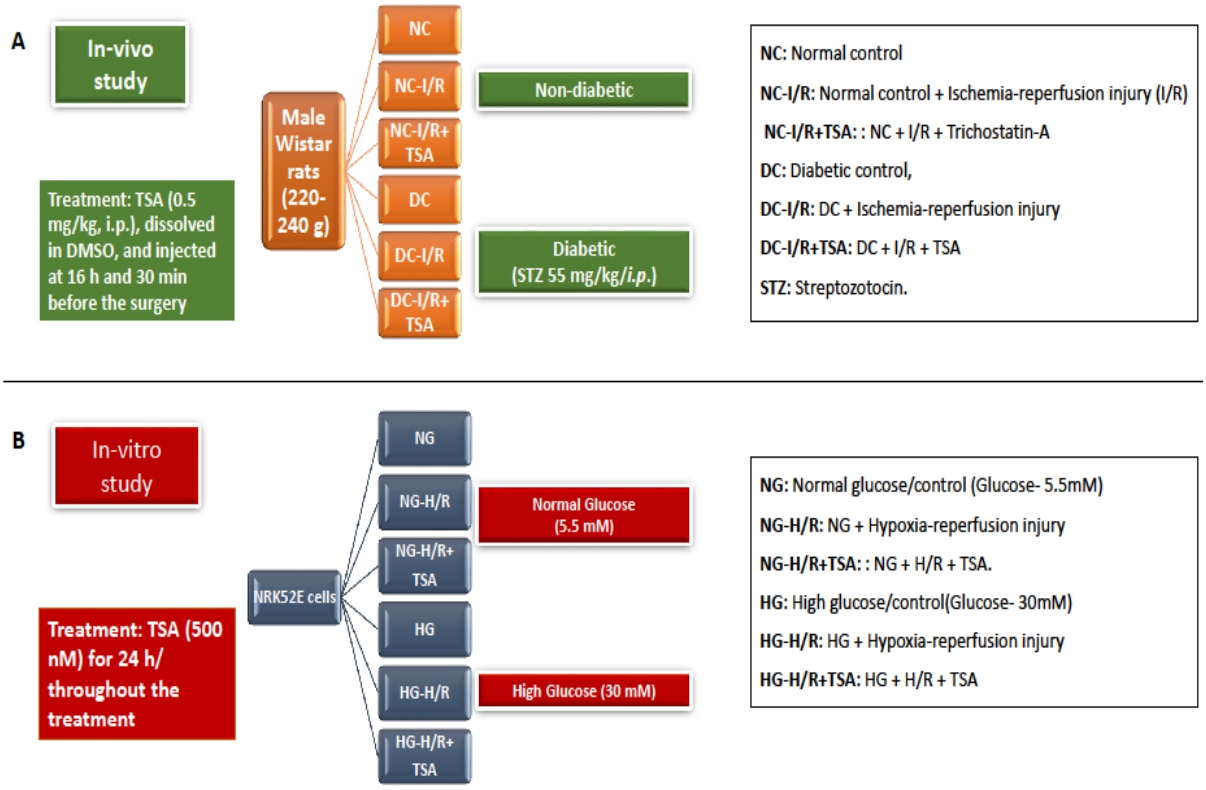


Figure 7: Schematic representation of the experimental groups and the study 1 design, both in-vitro and in-vivo

In-vitro study:

The NRK52E cells were grouped as follows: a) **NG/HG-** Normal glucose control (NG- 5.5 mM glucose)/ High glucose control (HG- 30mM glucose) served as respective controls, b) **NG-H/R/HG-H/R**- NG cells exposed to sodium azide followed by reperfusion/ HG exposed to sodium azide followed by reperfusion, c) **NG-H/R+TSA/HG-H/R+TSA**- NG-H/R cells treated with TSA (500nM)/ HG-H/R cells treated with 500nM TSA(Fig. 7B) (Hyndman et al., 2019; Tung et al., 2017). Treatment of TSA was maintained throughout the experiment. The dose of TSA (500nM) was selected based on the cell viability assay.

For Study 2: the objective of this study was to investigated ACE2 and Klotho regulation in AKI using ischemic Wistar rats and NRK52E cells under normal and hyperglycemic conditions (Fig. 8).

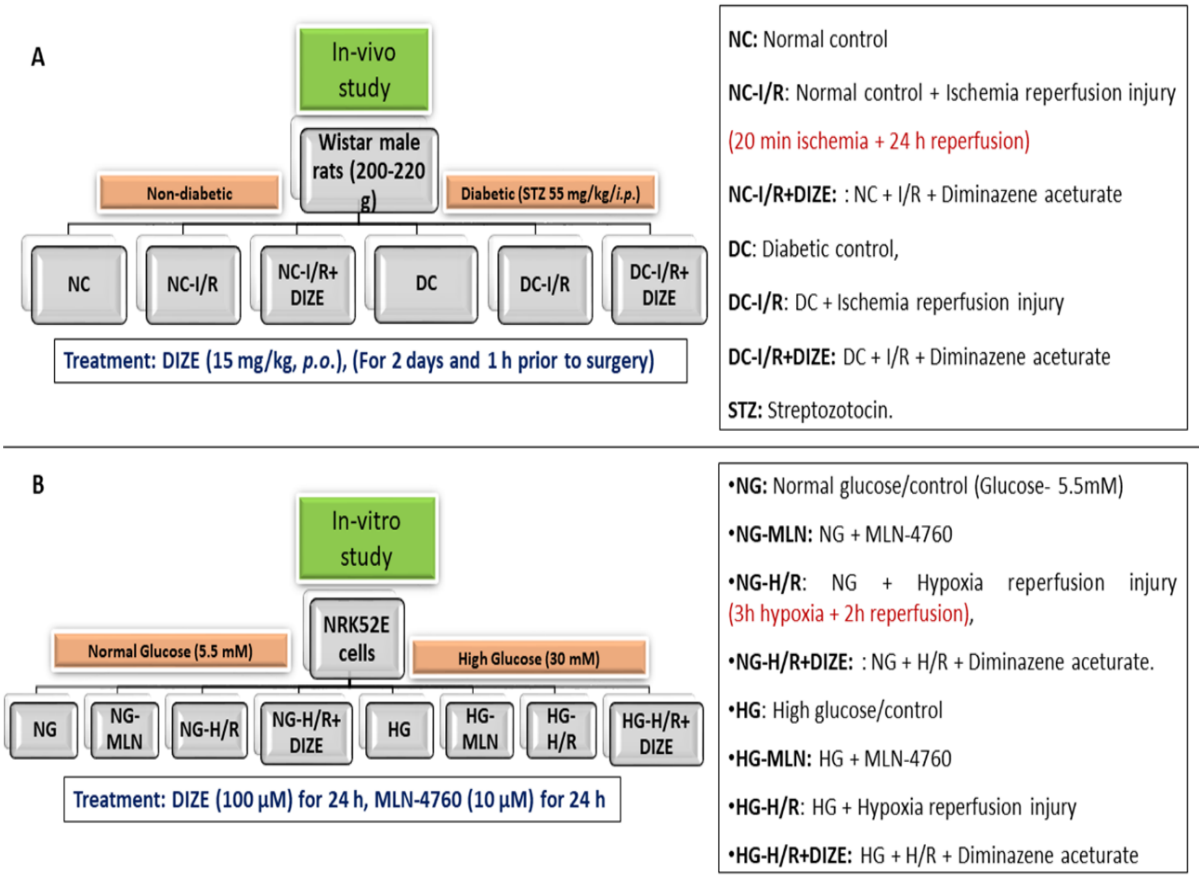


Figure 8: Schematic representation of the experimental groups and the study 2 design, both in-vitro and in-vivo

In-vivo study: Animals were divided into the following groups (Fig. 8A): a) NC/DC- normal control (NC)/diabetic control (DC) served as respective controls, b) NC-I/R/DC-I/R- NC rats subjected to 20 min IRI and 24 h reperfusion/ DC rats subjected to 20 min IRI and 24 h reperfusion, c) NC-I/R+DIZE/DC-I/R+DIZE- NC-I/R and DC-I/R rats treated with diminazene aceturate (DIZE) (15 mg/kg, *p.o.*). DIZE was dissolved in saline solution and administered for 2 days and 1 h before IRI.

In-vitro study: The NRK52E cells were divided into 6 and/or 8 groups (Fig. 8B). ACE2 activator- DIZE treatment was maintained throughout the experiments. The safe and effective dose of DIZE (100 µM) was chosen based on the literature and XTT assay.

For study 3: This study aimed to elucidate the affiliation between ER stress and Klotho regulation and develop a therapeutic approach against AKI-diabetes comorbidity (Fig. 9).

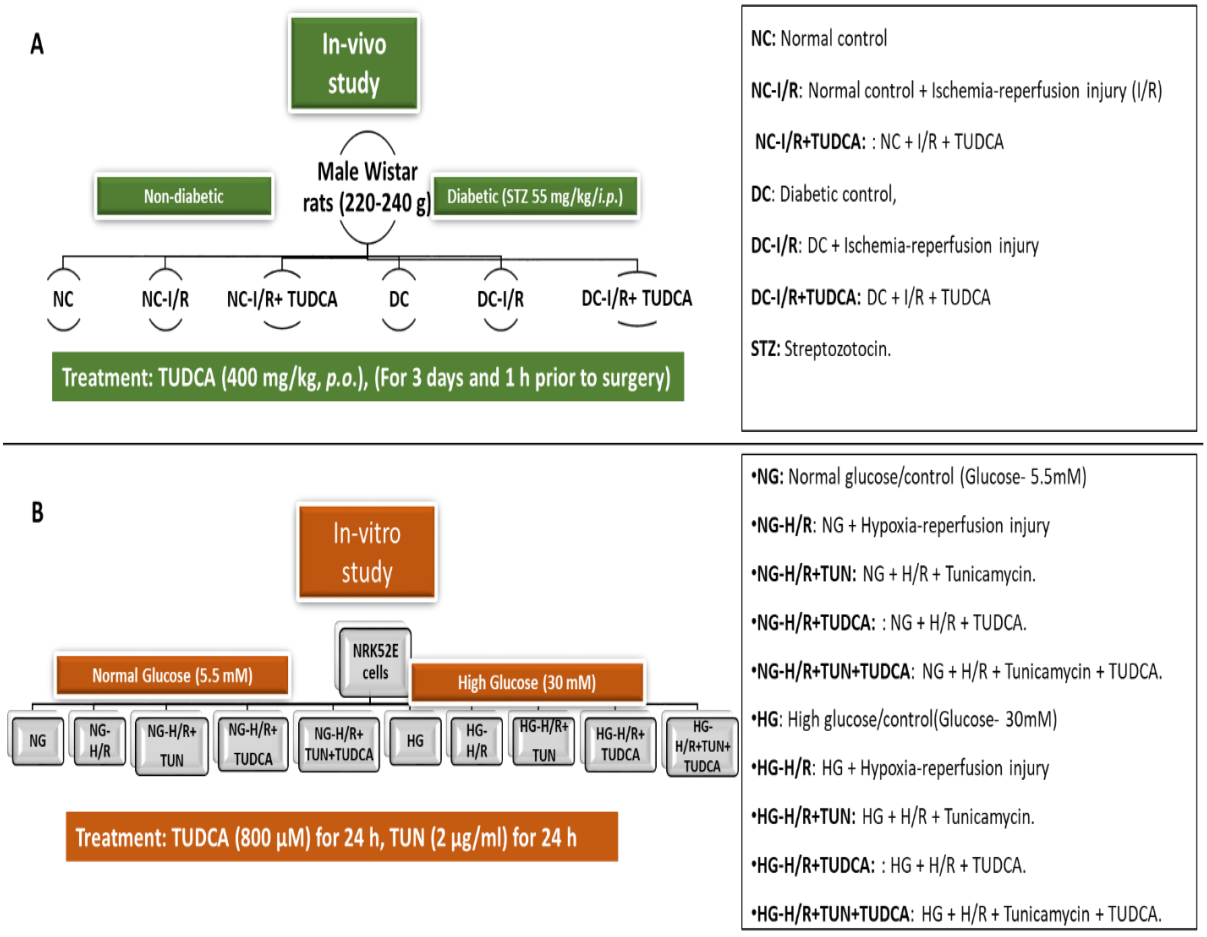


Figure 9: Schematic representation of the experimental groups and the study 3 design, both in-vitro and in-vivo

In-vivo study: Animals were divided into the following groups (Fig. 9A): a) NC/DC- normal control (NC)/diabetic control (DC) served as respective controls, b) NC-I/R/DC-I/R- NC rats subjected to 20 min IRI and 24 h reperfusion/ DC rats subjected to 20 min IRI and 24 h reperfusion, c) NC-I/R+TUDCA/DC-I/R+TUDCA- NC-I/R and DC-I/R rats treated with tauroursodeoxycholic acid (TUDCA) (400 mg/kg, *p.o.*). TUDCA was dissolved in saline solution and administered for 3 days and 1 h before IRI.

In-vitro study: The NRK52E cells were divided into 10 groups (Fig 9B). The TUN and TUDCA treatments were maintained throughout the experiments. The safe and effective dose of both drugs was chosen based on the literature and XTT assay.

4.3 Biochemical analysis of plasma and urine samples for assessment of renal functions

Before sacrifice, animals were kept in metabolic cages for 6 h for urine collection, followed by blood withdrawal and plasma separation using a centrifuge (5 min, 5000 g, 4 °C). Plasma glucose, creatinine, blood urea nitrogen (BUN), Klotho, neutrophil gelatinase-associated lipocalin (NGAL), ACE2, Ang-(1-7) were estimated using plasma, and kidney injury molecule-1 (KIM-1) was estimated using urine as per the standard protocols provided by the respective manufacturers of the ELISA kits.

4.4 Animal sacrifice and organ collection

Animal sacrifice was done by giving overdose of anesthesia. Kidney tissue was collected, washed with ice-cold normal saline and stored into the deep freezer (-80 ºC) to avoid further protein degradation. Further, the stored tissue was used for molecular biological experiments. For histopathology studies, the tissues were stored in 10% (v/v) formalin solution for fixation (Goru et al., 2017).

4.5 Cell viability assay

The NRK52E cells were incubated at a density of 1×10^4 cells/well in 96-well plate. After reaching to 70% confluency, cells were treated with different concentrations of drug for the next 24 h followed by HRI induction using sodium azide. After the HRI, MTT/XTT (10 µl of 5 mg/ml) was added to each well for the next 4 h, followed by a 20 min incubation with 150 µl DMSO (avoided when XTT was used) to dissolve the formazan crystals and absorbance was noted at 570 nm using a spectrophotometer.

4.6 Flow cytometry analysis: Annexin V-FITC/PI assay

To ensure the model development and differentiate the normal cells and apoptotic cells, fluorescence-activated cell sorting (FACs) was done using Annexin V-FITC kit (Kwan et al., 2016). The treated cells were trypsinized, collected and washed with cold PBS followed by centrifugation was done. Cells were resuspended using the binding buffer and incubated with FITC/PI stain for the prescribed period. After the incubation period FACs analysis was done using a flow cytometer (Beckman Coulter, USA).

4.7 RNA interference

Klotho targeted siRNA was used to silent the Klotho expression in NRK52E cells. The sense sequence utilized was 5′-GGCCUCAGAUAACCUUACUTT-3′ and the anti-sequence was 5′-AGUAAGGUUAUCUGAGGCCTT-3′ (Genecust, France). Lipofectamine 3000 reagent was used to transiently transfect the confluent NRK52E cells with Klotho siRNA for 24 h (Shrestha et al., 2022). This sample was collected and further used for the evaluating the expression of various proteins.

4.8 Proximal tubules isolation from the whole kidney

The kidneys were isolated from the respective animals and were placed in cold PBS (pH- 7.4), and tubular fractions were isolated using the percoll gradient centrifugation method with some modifications (Sharma and Gaikwad, 2020). Briefly, the kidney was minced and digested with collagenase type IV in PBS, with constant oxygenation until a uniform suspension was formed. The suspension was filtered through a nylon 250-µm sieve and centrifuged at 100 g for 1 min. The pellets were suspended and washed two times in ice-cold PBS. The pellet suspension in PBS was mixed thoroughly with 40% Percoll and centrifuged at 26,000 g for 30 min. Four distinct bands (B1-B4) were separated. The B4 band, highly enriched proximal tubular fraction, was carefully collected, suspended, and washed in ice-cold PBS. Thus, the obtained tubular fraction was assessed under the light microscope and used for further analysis.

4.9 Protein estimation

For sample preparation, we used isolated proximal tubular fraction or NRK52E cell lysates which were rinsed with ice-cold isotonic saline (0.9 % w/v NaCl). Later, the special lysis

buffer (LSB) for isolated PCT and RIPA buffer for NRK52E cell lysate were added and homogenization was done to extract the proteins. Further, centrifugation at 10,000 g for 15 min (4°C) was carried out to collect the supernatant. The supernatant was collected and the protein estimation was done using Bradford's reagent. Shortly, 10 µl of supernatant and 200 µl of 1x Bradford's reagents were mixed and incubated for 10 min, followed by protein estimation by reading the absorbance at 595 nm using a spectrophotometer.

Table 5: List of antibodies used throughout the study.

#	**Antibody Name**	**Dilution**	**Company**
1.	**Primary antibody against:** HDAC 1 (#34589), HDAC 2 (#57156), HDAC 3 (#85057), p-NFκ-β(S-536) (#3033), IκBα (#4814), cleaved PARP (#5625), cleaved Caspase-3 (#9664) H3K9Ac (#9649), H3K27Ac (#8173), p-eIF2α (#3597S), t-eIF2α (#5324S), BiP (#3183S), PERK (#3192S), c-Cas-7 (#8438T)	1:1000 (v/v)	Cell Signaling Technology (Danvers, MA, USA)
2.	**Primary antibody against:** Klotho (#PA521078)	1:1000 (v/v)	Invitrogen (California, USA)
3.	**Primary antibody against:** MCP-1 (sc-1785) β-actin (sc-4778)	1:1000 (v/v)	Santa Cruz Biotechnology (Dallas, Texas, USA)
4.	**Secondary antibodies:** Goat Anti-rabbit IgG (#7074) Horse anti-mouse IgG (#7076)	1:20000 (v/v)	Cell Signaling Technology (Danvers, MA, USA)
5.	**Secondary antibodies:** Rabbit anti-goat IgG (sc-2922) Goat anti-mouse IgG (sc-2005) Mouse anti-rabbit IgG (sc-2357)	1:20000 (v/v)	Santa Cruz Biotechnology (Dallas, Texas, USA)

4.10 Histology

Histology was performed as per the protocol described previously (Pandey et al., 2015). Briefly, the kidney tissue was fixed in 10% (v/v) formalin in phosphate-buffered saline and embedded in paraffin blocks. 5µm sections were taken by microtome and deparaffinized with xylene (2 times, three minutes each) followed by a rehydration process using gradient percentages of ethanol (100%, 90%, 80% 70%; 3 minutes each). Hematoxylin and eosin staining were performed as described previously (Goru et al., 2017). Slides were treated with hematoxylin and dehydrated in absolute alcohol followed by rehydration in distilled water. Then, slides were placed in eosin and dehydrated with gradient percentages of ethanol (90%, 100%; 2 times each), kept in xylene, and mounted using Di-N-Butyl Phthalate in Xylene (DPX) media. Thus, stained sections were evaluated for morphological alterations in tissues. At least 4-5 sections (one microscopy slide) from each tissue and a total of n=6 tissue from each group were observed; and were captured at 400x and 100x magnification by using a Zeiss microscope (model: Vert.A1) and Optika TCB5" microscope (Optika Research Microscope, Italy), respectively.

4.11 Immunohistochemistry

Immunohistochemistry was performed as described previously (Goru et al., 2017). Briefly; the kidney sections (5 µm) were taken from paraffin blocks and deparaffinized with xylene, followed by rehydration in gradient percentage of ethanol (100%, 95%, 70%; 3 minutes each) and distilled water. After, washing with PBS, slides were processed for antigen retrieval by heating in citrate buffer via microwave (10 mmol/L for 10 min). Leave the sections to cool down at room temperature (30 min) and washed with tris buffered saline (1X TBS). After then, sections were exposed to H_2O_2 (3%) for 15 minutes (to block endogenous peroxides), washed with tris buffered saline (1X PBS) prior to keeping the sections in blocking reagent BSA (5%) solution. After blocking, primary antibody incubation was done (12 h at 4°C) and then washed with PBS. Further, secondary antibody incubation was performed (1 h at room temperature), followed by detection with diaminobenzidine (DAB) as a chromogen. The slides were counterstained with hematoxylin, dehydrated with alcohol and xylene and mounted in DPX. Around 4-5 sections (one microscopy slide) from each tissue and a total of

n=6 from each group were observed. Then, the DAB-positive area was calculated using ImageJ software.

4.12 Histone protein isolation and immunoblotting

Histone proteins were isolated from proximal tubular fraction as previously described protocols (Malek and Gaikwad, 2019; Tikoo et al., 2008). Briefly, proximal tubular pellet was homogenized with Buffer A [12% w/v sucrose, 10mM EDTA, 10mM Tris, 1% PMSF (0.1M), 5mM NaCl, 0.1% NaBr (1M), adjusted pH-7.2], and further layered with Buffer B [15%w/v Sucrose, 10mM Tris, 0.1% NaB (1M), 10mM EDTA, 5mM NaCl, 1% PMSF (0.1M), and adjusted pH-7.4]. The whole composition was centrifuged at 4000 rpm to get a crude nuclear pellet. Thereafter, layering was done by adding 1% Triton-X solution followed by centrifugation. Collected pellets were washed with 12% (w/v) sucrose in buffer A in order to remove traces of Triton X. To get histones, obtained nuclear pellet was treated with low salt buffer containing concentrated HCl and sonicated accompanied by centrifugation at maximum speed. The supernatant was treated with 25% trichloroacetic acid to precipitate histone protein. The collected pellet was dissolved in water. Immunoblotting was performed using 7.5%, 10%, and 14% gel and the desired protein was resolved according to molecular weight (Shrestha et al., 2022). Obtained SDS gel was further transferred onto the nitrocellulose membrane, followed by overnight incubation with primary antibodies (1:1000 (v/v) dilutions). As secondary treatment over, proteins were detected using the electrochemiluminescence (ECL) system and Hyperfilm. Densitometric analysis using ImageJ software was done to quantify immunoblots.

4.13 RNA isolation and Real Time-Polymerase Chain Reaction (RT-PCR)

RNA was isolated from proximal tubules by using TRIzol reagents and purified by Ambion™ PureLink™ RNA Mini Kit (Thermo Fisher Scientific, MA, USA) (Goru SK et al., 2017). qRT-PCR was performed using specific primers designed and produced by Eurofins, India. Briefly, 5 µg of RNA was taken and incubated with 1 µl (2U) of recombinant DNase-I for 30 min at 37°C [Ambion™ Recombinant DNase-I (RNase-free), Life Technologies, USA] in order to remove the single or/and double-stranded DNA, chromatin and RNA-DNA hybrids exist in the sample. Allowing the samples to heat at 75°C along with 5mM of EDTA were inactivated the DNase-I. Further, cDNA was synthesized by utilizing a cDNA kit

(GeneSure™ First Strand cDNA Synthesis Kit, Puregene, Genetix brand, USA). The samples were incubated at a gradient temperature cycle i.e. 25°C for 5 min, 42°C for 60 min, and inactivation at 70°C for 5 min. A quantitative real-time polymerase chain reaction of the samples was done according to the lab protocol on Light Cycler® 96 Real-Time PCR System using the Fast Start Essential DNA Green Master. Finally, the results were analyzed by Light Cycler® Software (Roche, Mannheim, Germany). After amplification, a melting curve analysis was done to ensure the specificity of the reaction. Enrichment of targeted mRNA was normalized against 18s rRNA contents. Experiments were carried out in triplicate for each sample and results are expressed as fold changes over respective controls (Malek and Gaikwad, 2019).

4.14 Statistical analysis

Experimental values are represented as mean ± S.D. and n refers to the number of samples studied. Moreover, statistical comparison was performed using one-way ANOVA, and if the F value was significant, multiple comparisons were performed by Tukey's Multiple Comparison post hoc test using GraphPad Prism software version 8.00 (San Diego, CA, USA). Data for which $p < 0.05$, were considered statistically significant.

5. Results

5.1 Epigenetic restoration of endogenous Klotho expression alleviates acute kidney injury-diabetes comorbidity

5.1.1 HDACs inhibition reestablished hyperglycemia exaggerated Klotho deficiency and renal dysfunctions in diabetic and non-diabetic ischemic rats

Herein, to understand the effect of HDAC inhibition on Klotho regulation and AKI progression, the biochemical analysis was performed. The parameters like, BUN, plasma creatinine, plasma Klotho and urinary KIM-1was checked (Fig. 10). Further, plasma glucose level was checked in STZ induced diabetes rats wherein TSA did not show any effect on glucose level (Fig. 10A).

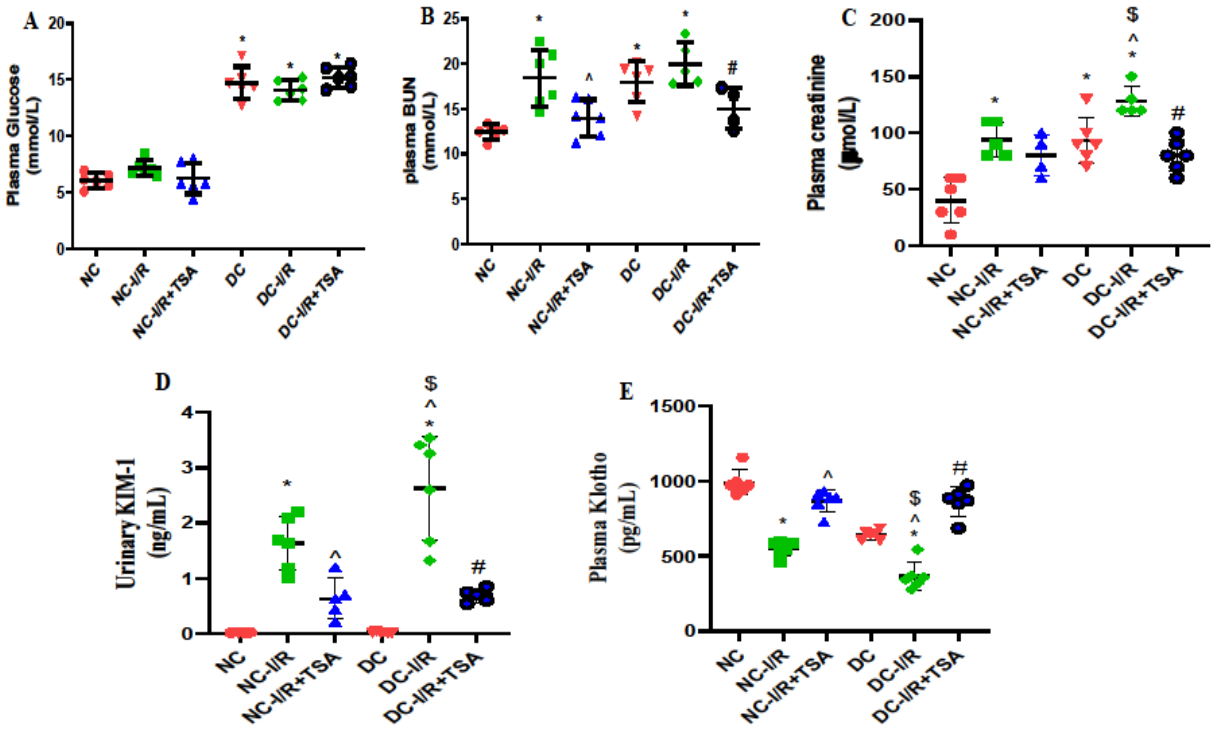


Figure 10: Effect of HDACs inhibition on renal dysfunction in diabetic and non-diabetic ischemic rats. A-E*: Above scattered plots are representing the plasma glucose (A), blood urea nitrogen (BUN) (B), plasma creatinine (C), urinary KIM-1 (D) and plasma Klotho (E), where the data are represented as mean ± SD (n=6). For statistical comparison, one-way ANOVA with Tukey's multiple comparison test was used where (*) p < 0.05, vs NC; (^) p <0.05 vs NC-I/R; ($) p < 0.05 vs DC; (#) p < 0.05 vs DC-I/R.*

After the IRI, the levels of BUN (Fig. 10B), plasma creatinine (Fig. 10C), KIM-1 (Fig. 10D) was increased, and Klotho levels (Fig. 10E) were decreased significantly ($p < 0.05$) compared to non-IRI animals. However, in IRI with diabetes animals the levels of plasma creatinine, urinary KIM-1 was further elevated, and the level of Klotho was reduced, significantly ($p < 0.05$) compared to non-diabetic IRI animals (Fig. 10). Impressively, after the treatment with TSA (0.5 mg/kg/*i.p.*) altered level of these biomarkers in non-diabetic and in diabetic, IRI groups were restored (Fig. 10). This result clarifies that hyperglycemia aggravated AKI via Klotho downregulation, which was restored upon treatment with an HDACs inhibitor, TSA.

5.1.2 Kidney specific HDACs inhibition improved morphological characteristics of NRK52E cells and proximal tubules of the ischemic kidney

NRK52E cells were treated for 24 h with different concentrations of TSA ranging from 0nM-1000nM. Based on the MTT assay we have chosen 500nM of TSA as a submaximal dose to treat the NRK52E cells (Fig. 11A).

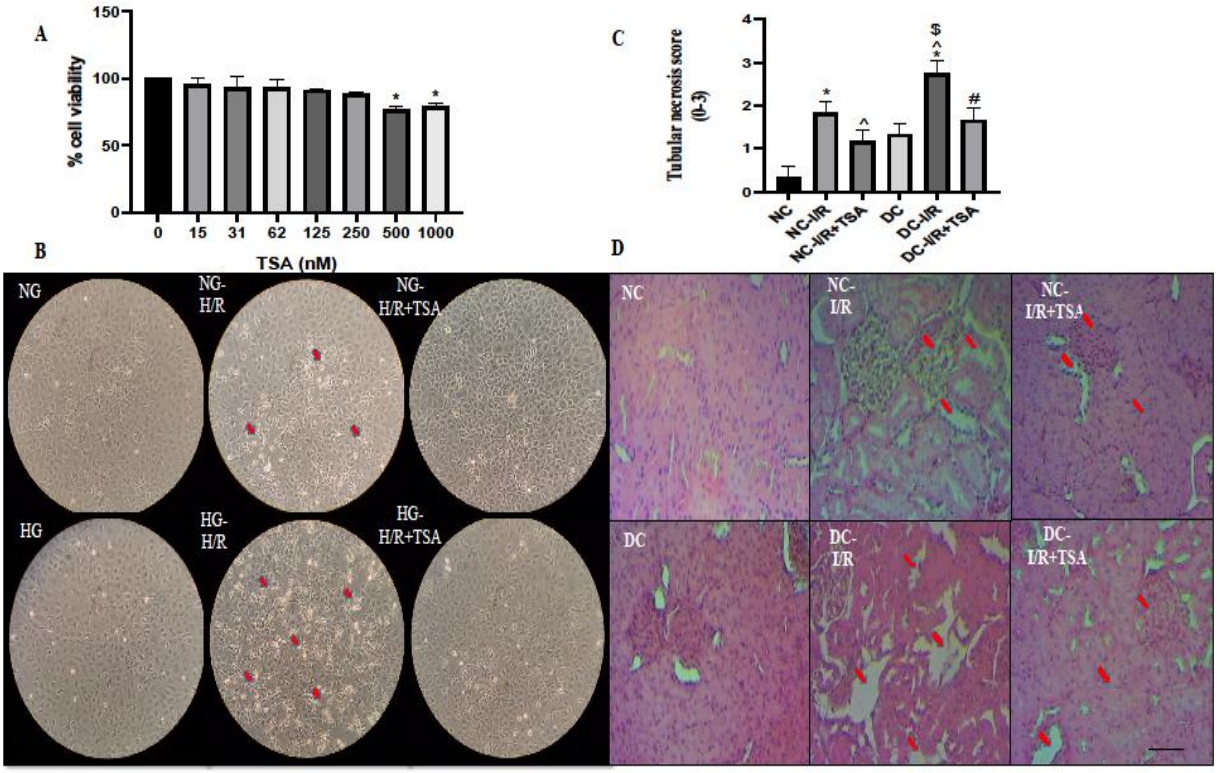


Figure 11: Effect of HDACs inhibition on cell viability and morphology of the ischemic kidney.

A-D: NRK52E cells were treated for 24 h with different concentration of TSA ranging from 0-1000nM (A). To check the morphological changes in NRK52E cells, the microscopic evaluation was done (original magnification 200x and scale bar- 50 μM) (B). Histological examination was done by H and E staining. The percentage of tubular injury was analysed semi-quantitatively (C). Representative images of kidney sections (D) (original magnification 400x and scale bar- 50 μM). At least 4-5 images from each stained kidney section and a total of six different kidneys per group were observed by a blinded observer for tubular dilatation and injury (indicated by red arrow). Data are represented as mean ± SD. one-way ANOVA with Tukey's multiple comparison test was used where (*) $p < 0.05$, vs NC/NG; (^) $p < 0.05$ vs NC-I/R/NG-H/R; ($) $p < 0.05$ vs DC/HG; (#) $p < 0.05$ vs DC-I/R/HG-H/R.

Microscopic analysis of cultured tubular cells revealed that sodium azide induced hypoxia reperfusion injury (HRI) in the presence or absence of hyperglycemia (30mM) persuaded cells morphology such as condensed structure, loss of cell membrane and floating death cells (Fig. 11B). Moreover, under the high glucose condition, HRI resulted in further distinction in NRK52E cells morphology compared to the low glucose (5.5 mM) HRI group. However, these changes were effectively restored upon TSA treatment in both low glucose HRI and high glucose HRI groups. Histological examination of the kidney using H and E staining showed that IRI leads to pathological changes in kidney structure that exhibit the feature of AKI including tubular dilatation and injury (Fig. 11C). Notably, diabetic ischemic animals showed more tubular dilatation and tubular necrosis (shown by red arrow) compared to ischemic and normal animals (Fig. 11D). However, these pathological changes in kidney histology were efficiently reduced after TSA treatment. Thus, hyperglycemia further intensified the IRI/HRI induced AKI as observed in microscopic images *in-vitro* and *in-vivo*, which was preserved by TSA.

5.1.3 Inhibition of kidney-specific HDACs restored the hyperglycemia exaggerated Klotho deficiency, altered HDACs and histones levels in AKI.

The biochemical and histological results of the present study revealed that hyperglycemia aggravates kidney damage and TSA administration has shown protective action against it. Post-transcriptional histone modifications play an important role in Klotho regulation and AKI progression. However, to further understand this at the molecular level we performed

western blotting analysis. Densitometric analysis of western blots revealed that HRI/IRI downregulates endogenous Klotho level and promotes the expression of kidney-specific histone deacetylase in NRK52E cells and kidney of the ischemic rats (Fig. 12A, H). However, in presence of hyperglycemia the significant increase in ($p < 0.05$) the expressions of HDACs 1, 3, 6 (Fig. 12B, D, E) *in-vitro* and HDACs 1, 2, 3, 6 (Fig. 12I, J, K, L) *in-vivo* was observed. Also, decline in the Klotho level (Fig. 12F, *in-vitro* and Fig. 12M, *in-vivo*) was seen when compared to normal and IRI groups.

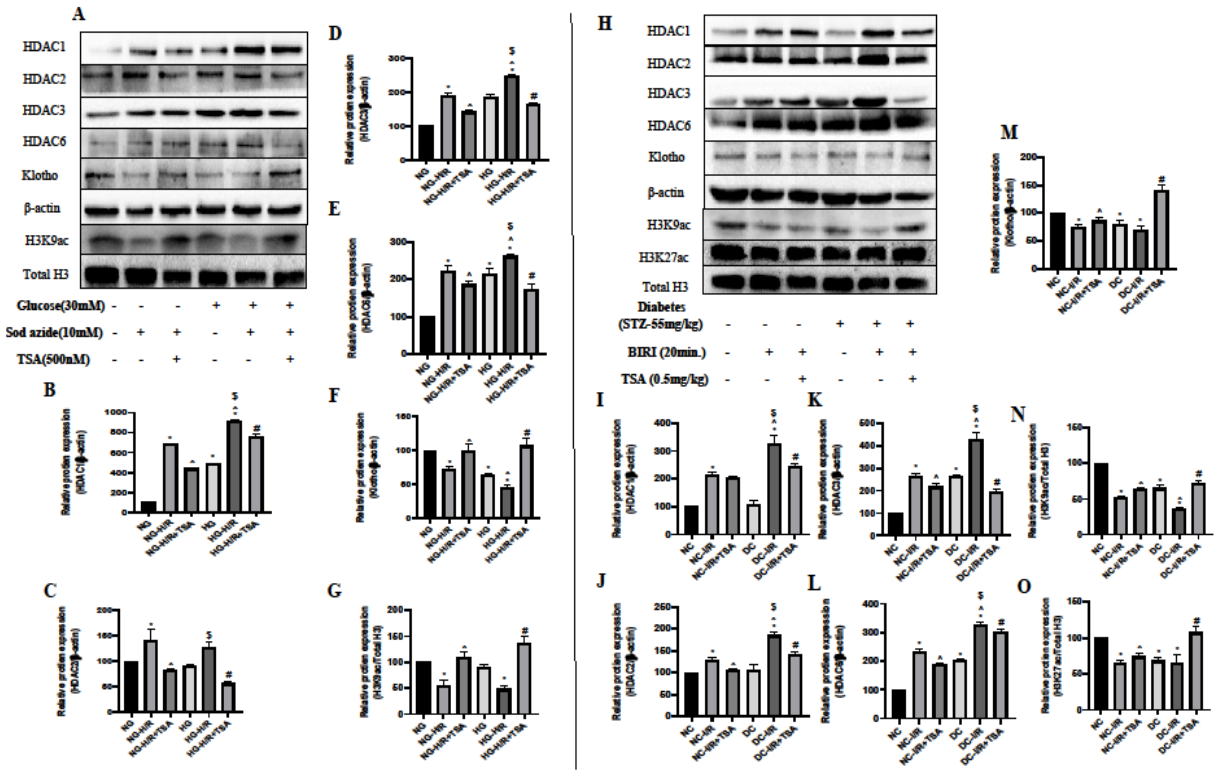


Figure 12: Effects of HDACs inhibition on expression of kidney specific HDACs, H3K9Ac, H3K27Ac and Klotho proteins in-vitro and in-vivo. *A-O: Protein levels of HDAC1 (B. I), HDAC2 (C, J), HDAC3 (D, K), HDAC6 (E, L), H3K9Ac (G, N), H3K27Ac (O) and Klotho (F, M) were checked in NRK52E cell lysate and isolated proximal tubules homogenate (n=3). Data are represented as mean ± SD from three independent experiments. One-way ANOVA with Tukey's multiple comparison test was applied for statistical comparison. (*) $p < 0.05$, vs NC/NG; (^) $p < 0.05$ vs NC-I/R/NG-H/R; ($) $p < 0.05$ vs DC/HG; (#) $p < 0.05$ vs DC-I/R/HG-H/R.*

More interestingly, HDACs inhibition by TSA treatment efficiently ($p < 0.05$) restored the endogenous Klotho level in HRI and IRI under hyperglycemic as well as non-hyperglycemic conditions (Fig. 12F, *in-vitro* and Fig. 12M, *in-vivo*). This indicates that HDACs inhibition restores endogenous Klotho levels and improves kidney functions. The above results demonstrate that HRI or IRI increased the HDACs expressions which were further exaggerated in presence of hyperglycemia. However, we further checked the effects of HRI/IRI on the expression of histone- H3K9Ac and H3K27Ac under hyperglycemic and non-hyperglycemic conditions.

In contrast, we observed that the protein level of H3K9Ac in NRK52E cells (Fig. 12G), and the protein level of H3K9Ac, and H3K27Ac histones in ischemic rats, were suppressed (Fig. 12N, O). Moreover, in the hyperglycemic IRI group, the protein level of H3K9Ac was significantly decreased ($p < 0.05$) compared to the non-hyperglycemic IRI group (Fig. 12N). Intriguingly, following TSA treatment the levels of H3K9Ac and H3K27Ac was significantly restored ($p < 0.05$) (Fig 12G, N, O). This observation suggests that in presence of hyperglycemia IRI/HRI lead to a significant increase in expression of kidney specific HDACs and reduction in H3K9Ac and H3K27Ac protein level along with Klotho downregulation.

5.1.4 Inhibiting the kidney specific HDACs diminished the hyperglycemia augmented Klotho deficiency and apoptosis in ischemic AKI

Microscopic and histological studies indicate that HRI and IRI alter the morphology of cells and kidney tissue, respectively. Hence, we contemplate that apoptosis may involve in alteration of their morphology. To determine this, we performed flow cytometric analysis of NRK52E cells after the HRI (Fig. 13A). FACS imaging revealed that HRI significantly ($p < 0.05$) induced tubular apoptosis, where 7.41% and 9.44% apoptotic cells were observed in low glucose and high glucose HRI groups, respectively. However, the percentage of apoptotic cells in control and TSA treated groups were insignificant (Fig. 13A) indicates that TSA may have anti-apoptotic activity via Klotho regulation. An expression of caspase family proteins signifies the process of apoptosis. The western blotting of NRK52E cell lysate showed an elevated expression ($p < 0.05$) of cleaved-PARP, cleaved-caspase 9 in HRI groups (Fig. 13E) compared to the control group. Also, the RT-PCR report revealed that the caspase 7 mRNA expression was increased in HRI groups (Fig. 13C). Also, the expression of cleaved-PARP

(Fig. 13D), and cleaved-caspase 9 (Fig. 13F) were significantly ($p < 0.05$) higher in the high glucose HRI group when compared to the low glucose HRI group. An expression of these proteins was diminished upon TSA treatment ($p < 0.05$). A similar analysis was performed for kidney lysate samples (Fig. 13H). An expression of cleaved-PARP (Fig. 13I), cleaved-caspase 3 (Fig. 13J) and cleaved-caspase 9 (Fig. 13K) were increased and Klotho level (Fig. 13L) was decreased significantly ($p < 0.05$) in the IRI group compared to respective controls. Here also, the expression of the aforementioned apoptotic proteins was significantly higher ($p < 0.05$) in the diabetic IRI groups compared to non-diabetic IRI groups. Fascinatingly, TSA restored the Klotho level and decreased the expression of apoptotic proteins markedly ($p < 0.05$) in both diabetic as well as non-diabetic IRI groups. This indicates that apoptosis was involved in AKI pathogenesis where it may have a relation with Klotho regulation.

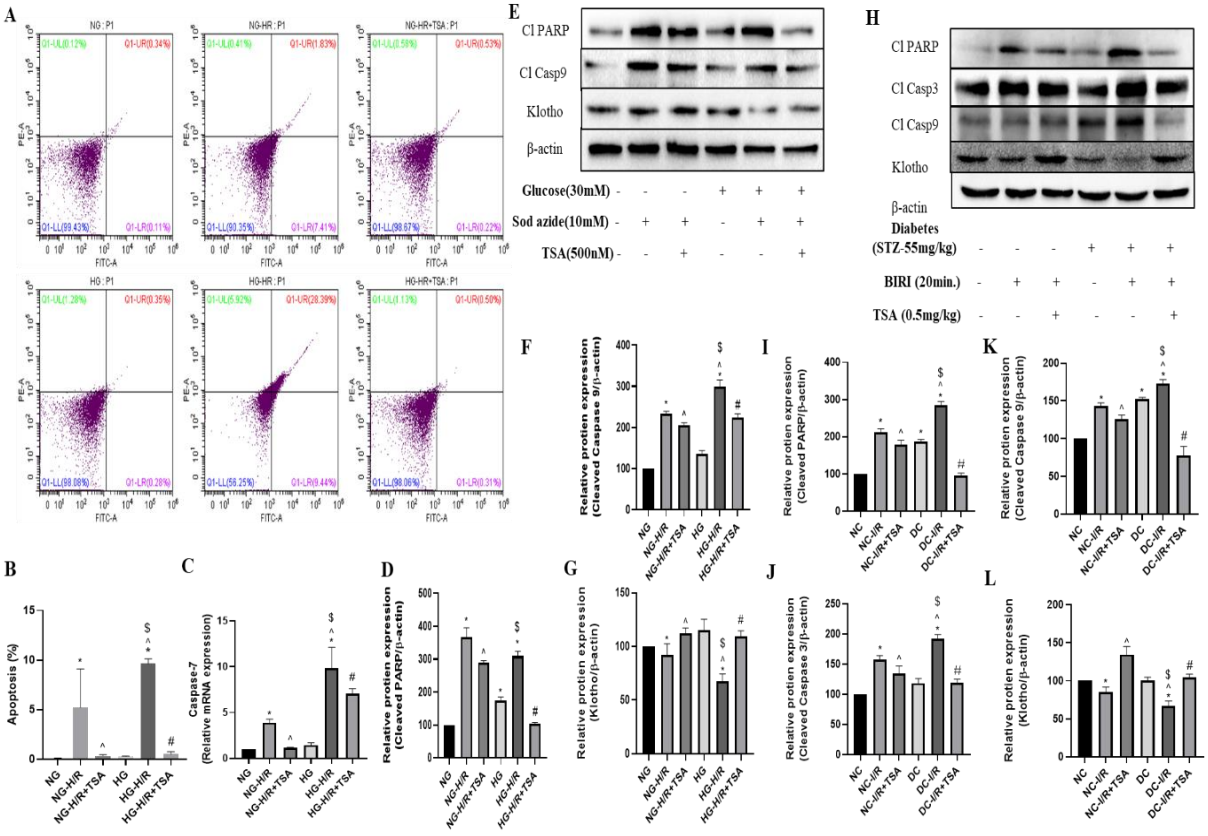


Figure 13: Effects of HDACs inhibition on apoptosis and Klotho level in-vitro and in-vivo under hyperglycemic and non-hyperglycemic conditions.

A-L: Flow cytometric detection of apoptosis (%) in NRK52E cells (A, B). Quantitative RT-PCR was performed for evaluation of caspase-7 mRNA expression in NRK52E cells (C). Western blot analysis was conducted for assessment of Klotho and apoptotic protein expression in NRK52E (E) and isolated proximal tubular lysate (H). β-actin was used as a loading control. Proteins such as; cl-PARP (D), cl-casp9 (F), Klotho (G) in NRK52E cells and cl-PARP (I), cl-casp3 (J), cl-casp9 (K), and Klotho (L) in tubular lysate of the kidney (n=3). All immunoblots were quantified with the help of densitometric analysis. Data are represented as mean ± SD from 3 independent experiments. One-way ANOVA with Tukey's multiple comparison test was applied for statistical comparison. (*) p < 0.05, vs NC/NG; (^) p < 0.05 vs NC-I/R/NG-H/R; ($) p < 0.05 vs DC/HG; (#) p < 0.05 vs DC-I/R/HG-H/R.

5.1.5 HDAC inhibition prevented hyperglycemia exacerbated inflammatory signaling pathway along with Klotho upregulation in hypoxic/ischemic renal injury

An increase in levels of inflammatory mediators is often seen in kidney injury. However, the impact of the TNFα- IKK-α/β-NF-κB axis on Klotho regulation or vice-versa in AKI under the hyperglycemic condition is entirely unknown.

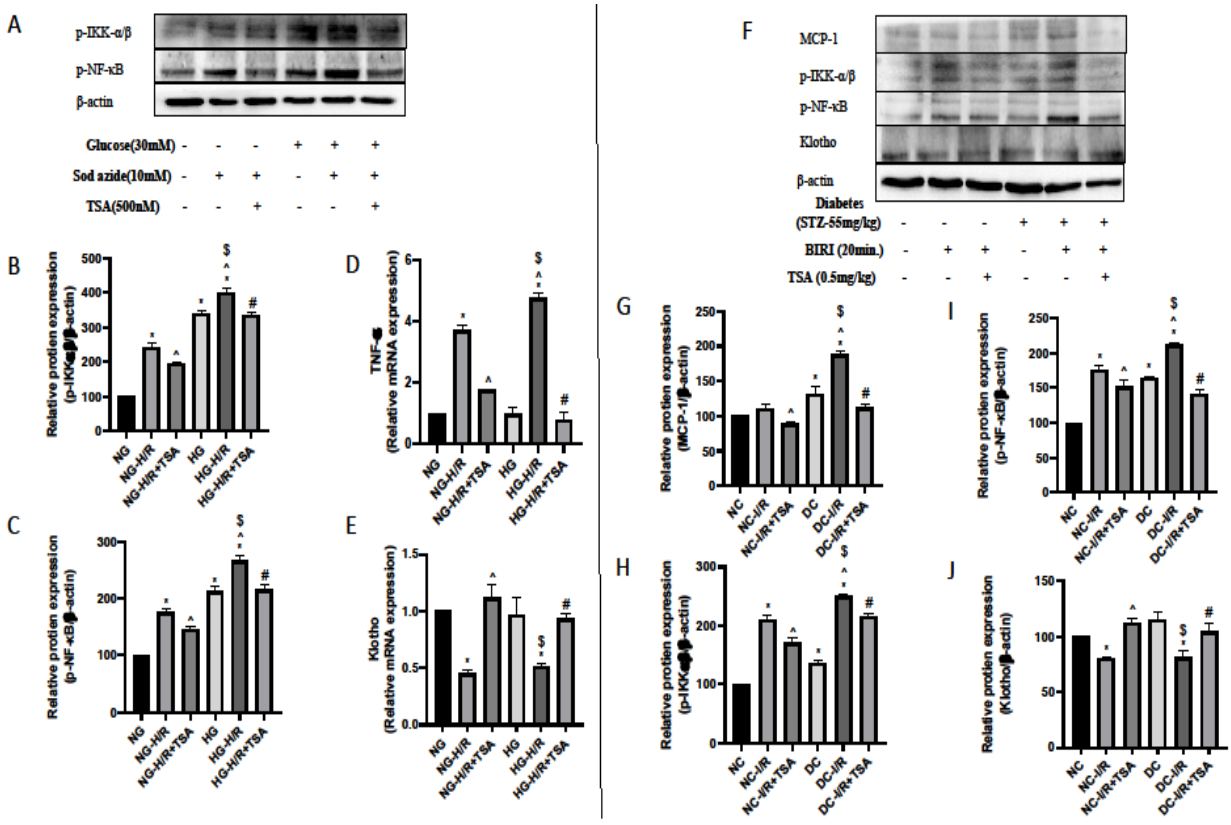


Figure 14: Effects of HDACs inhibition on inflammation and Klotho in-vitro and in-vivo under hyperglycemic and non-hyperglycemic conditions. *A-J: Protein levels p-IKK-α/β (B), p-NF-κB (C) and mRNA expression for Klotho (E) and TNF-α (D) were estimated in NRK52E cell lysate. The protein estimation for MCP-1 (G), p-IKK-β/α (H), p-NF-κB (I), and Klotho (Fig. J) were checked using proximal tubules homogenate (n=3). All immunoblots were quantified by densitometric analysis. Data are represented as mean ± SD from three independent experiments. One-way ANOVA with Tukey's multiple comparison test was applied for statistical comparison. (*) p < 0.05, vs NC/NG; (^) p <0.05 vs NC-I/R/NG-H/R; ($) p < 0.05 vs DC/HG; (#) p<0.05 vs DC-I/R/HG-I/R.*

Also, TSA is reported for anti-inflammatory activity in renal fibrosis but not reported in AKI. Our study demonstrates that HRI/IRI induced AKI seen with augmented level ($p < 0.05$) of inflammatory markers such as TNF-α, IKK-α/β, and MCP-1, p-NF-κB, proteins (Fig. 14A, F). More importantly, their expressions ($p < 0.05$) was further increased in a diabetic ischemic group. On the parallel side, we also noticed that Klotho mRNA and protein expression has shown an inverse relationship with inflammatory proteins (Fig. 14E, J, respectively). Interestingly, TSA treatment re-established the Klotho level alongside inhibited the TNFα-IKK- α/β-NF-κB axis in renal tubular cells.

5.1.6 HDACs inhibition provides reno-protection by upregulating the Klotho expression in AKI under hyperglycemic and non-hyperglycemic condition

Based on the above findings we hypothesized that TSA might be producing these reno-protective effects via Klotho restoration. Hence, to further understand this notion and the role of Klotho in AKI development we performed Klotho knockdown (Klotho siRNA 100nM conc.) in NRK-52E cells under the high glucose and normal glucose conditions (Fig. 15G). After the Klotho siRNA transfection, Klotho expression was significantly reduced (Fig. 15A, G). Immunoblotting results revealed that, after the Klotho knockdown, an expression of inflammatory protein- NF-κB (Fig. 15B) and apoptotic protein- cleaved-PARP (Fig. 15C) remained elevated in TSA treated group however it was reduced in the control group (scrambled siRNA). Moreover, Klotho knockdown does not alter the TSA induced HDACs inhibition (Fig. 15D, F). This outcome indicates that Klotho is an essential factor for the TSA mediated reno-protection in AKI.

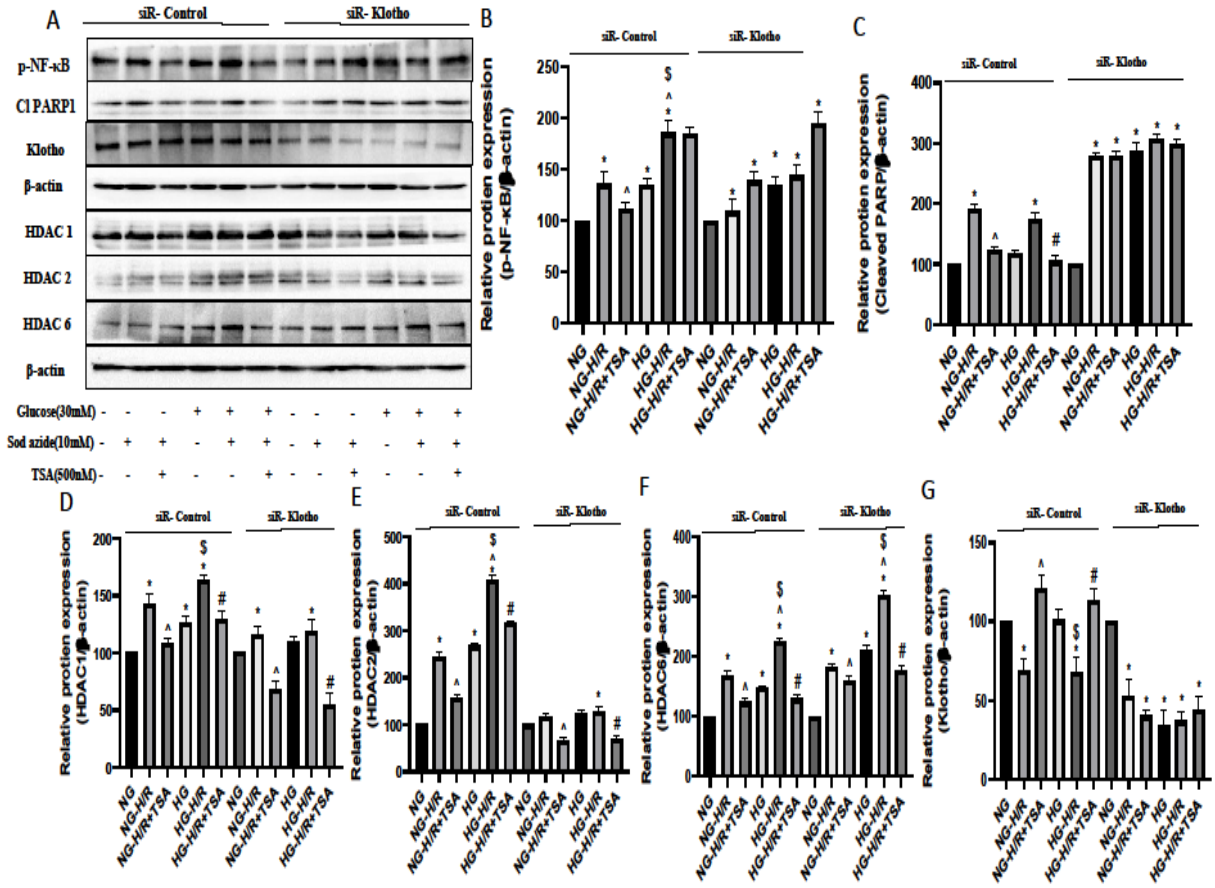


Figure 15: Klotho is essential for TSA-mediated reno-protection in NRK52E cells under hyperglycemic and non-hyperglycemic conditions. *A-G: The protein estimation for p-NF-κB (B), p-IKK-α/β (C), HDAC1 (D), HDAC2 (E), HDAC6 (F) and Klotho (G) were checked in NRK-52E cell lysate (n=3). All immunoblots were quantified with the help of densitometric analysis. Data are represented as mean ± SD from three independent experiments. One-way ANOVA with Tukey's multiple comparison test was applied for statistical comparison. (*) p < 0.05, vs NC/NG; (^) p < 0.05 vs NC-I/R/NG-H/R; ($) p < 0.05 vs DC/HG; (#) p < 0.05 vs DC-I/R/HG-H/R.*

5.2 Klotho restoration via ACE2 activation: A potential therapeutic strategy against acute kidney injury-diabetes comorbidity.

5.2.1 Effect of ACE2 activation on Klotho and kidney function parameters in diabetic and non-diabetic ischemic rats.

The ACE2 activator (DIZE, 15 mg/kg, *p.o.*) was administered for 2 days and 1 h before IRI in diabetic and non-diabetic rats.

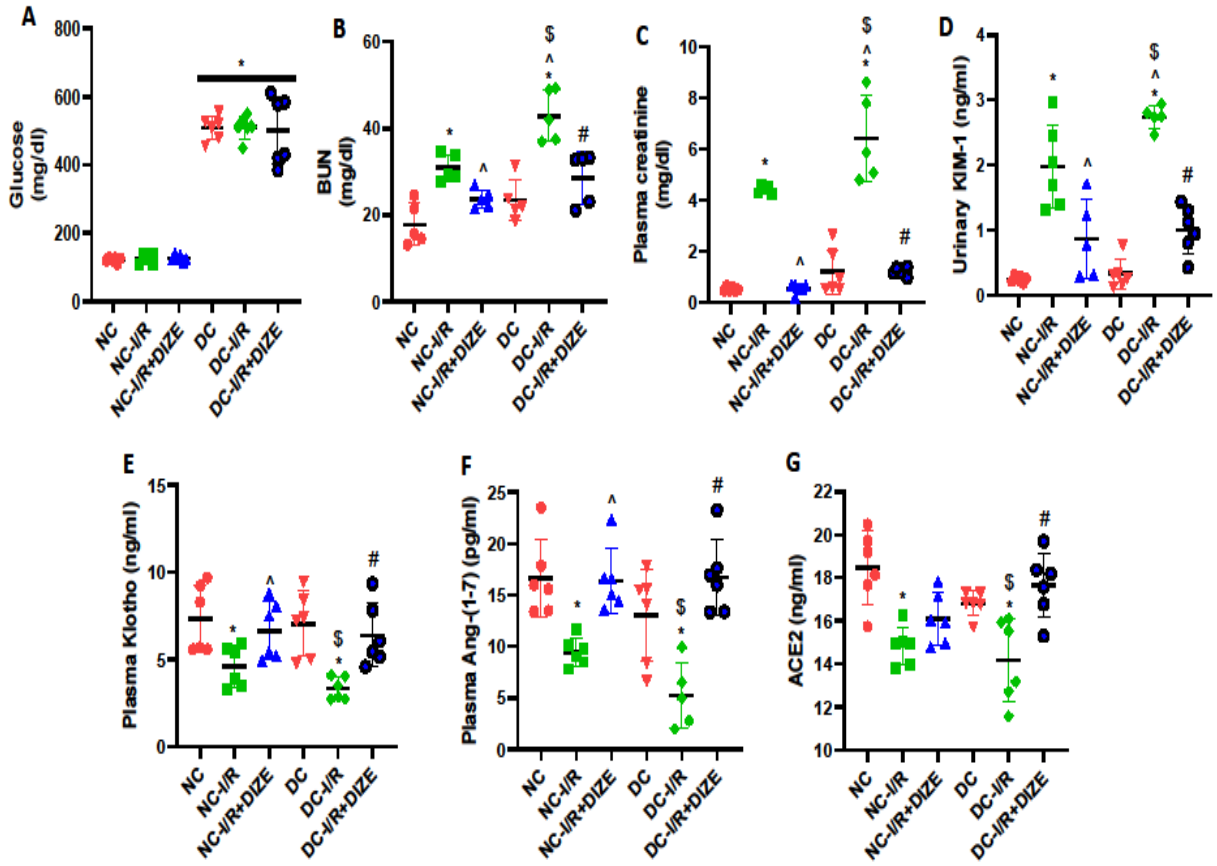


Fig. 16. Effect of ACE2 activation on plasma and urinary biochemical parameters. *A-G: Given scattered plots represents the level of, plasma glucose (A), BUN (B), plasma creatinine (C), uKIM-1 (D), plasma Klotho (E), plasma Ang-(1-7) (F) and tissue ACE2 (G), where the all experimental values are represented as mean ± SD (n=5-6). The one-way ANOVA followed by Tukey's multiple comparison test was used for statistical comparisons, where (*) $p < 0.05$, vs NC; (^) $p < 0.05$ vs NC-I/R; ($) $p < 0.05$ vs DC; (#) $p < 0.05$ vs DC-I/R.*

Plasma glucose levels were significantly higher in diabetic rats than in non-diabetic rats. In contrast, DIZE treatment did not change glucose levels (Fig. 16A). In the IRI groups (NC-I/R and DC-I/R), BUN (Fig. 16B), plasma creatinine (Fig. 16C), and urinary KIM-1 levels (Fig. 16D) were significantly increased ($p < 0.05$) and plasma Klotho (Fig. 16E), Ang-(1-7) (Fig. 16F), and renal ACE2 (Fig. 16G) levels were significantly decreased ($p < 0.05$) when compared to NC and DC (without IRI). However, BUN (Fig. 16B), plasma creatinine (Fig. 16C), and urinary KIM-1 (Fig. 16D) levels were significantly higher ($p < 0.05$) in the diabetic-ischemic group (DC-I/R) as compared to the non-diabetic ischemic group (NC-I/R).

These findings indicate that IRI leads to the development of AKI, in which hyperglycemia is a risk factor. Remarkably, DIZE treatment resulted in a significant decrease in BUN, plasma creatinine, and urinary KIM-1 levels and restoration of renal ACE2, systemic Ang-(1-7), and Klotho levels, suggesting that ACE2 activation may play an important role in Klotho regulation and thus renoprotection in AKI.

5.2.2 Effect of ACE2 activation on kidney histopathology of diabetic and non-diabetic ischemic rats

To further check the effect of ACE2 activation and IRI on the tissue-specific expression of inflammatory markers, we performed the IHC of kidney sections (Fig. 17A, B, D, E). After IRI-induced AKI, the expression of MCP-1 (Fig. 17A) and p-NF-kB (Fig. 17B) were significantly increased compared to the control groups. The expression of these proteins was further increased in presence of diabetes. However, in the treatment groups, the expression of MCP-1 and p-NF-kB decreased significantly (Fig. 17A, B) ($p < 0.05$). Furthermore, we also examined the kidney histology and measured kidney injury scores by performing H and E staining (Fig. 17C, F). Histological analysis revealed that IRI led to increased tubular dilatation and injury in the tubules and outer cortex of the kidney. However, in the DC-I/R group the severity of kidney injury was further increased (Fig. 17C, F). Interestingly, in the DIZE-treated group the kidney structure was normal. These findings suggest that IRI increases inflammation and kidney injury, which was restored by ACE2 activation.

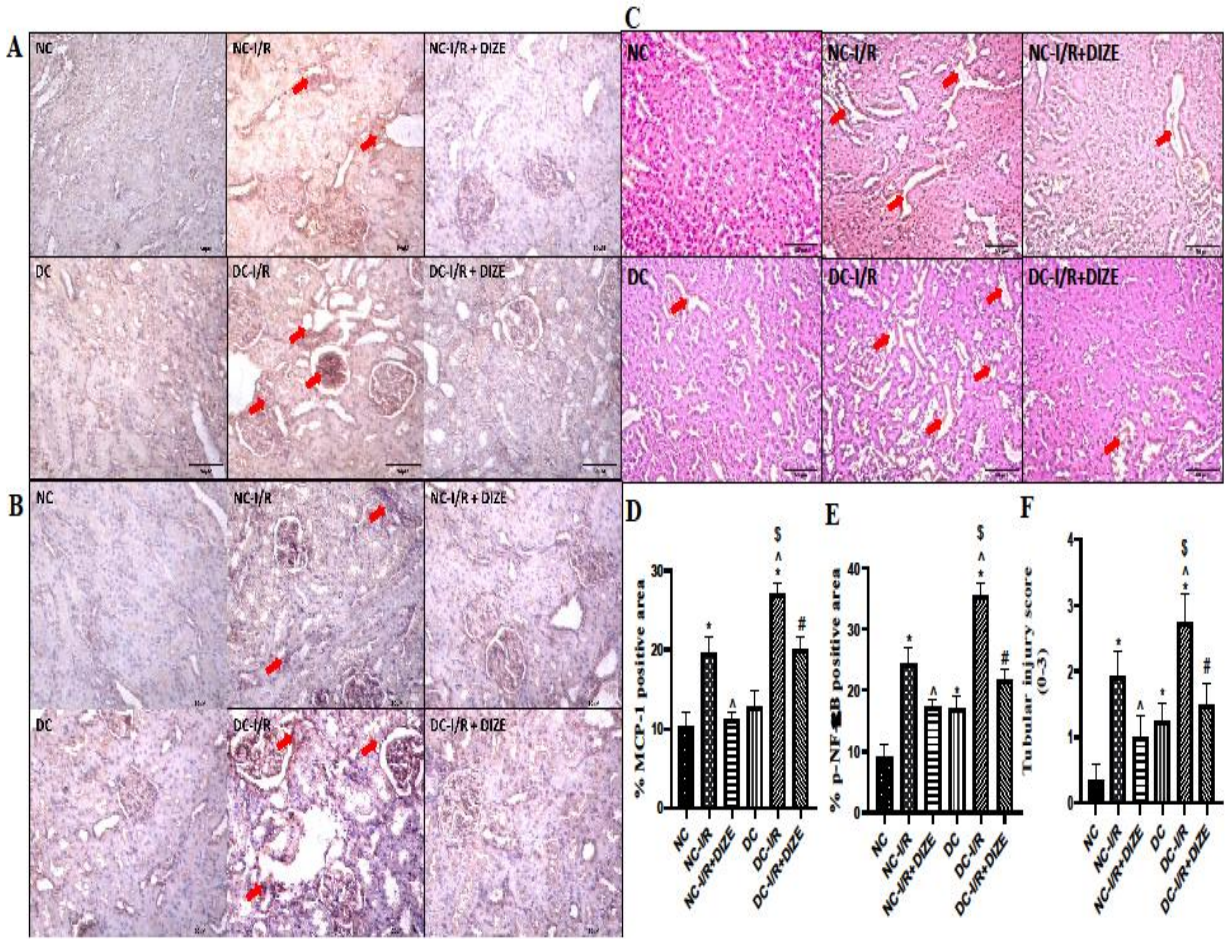
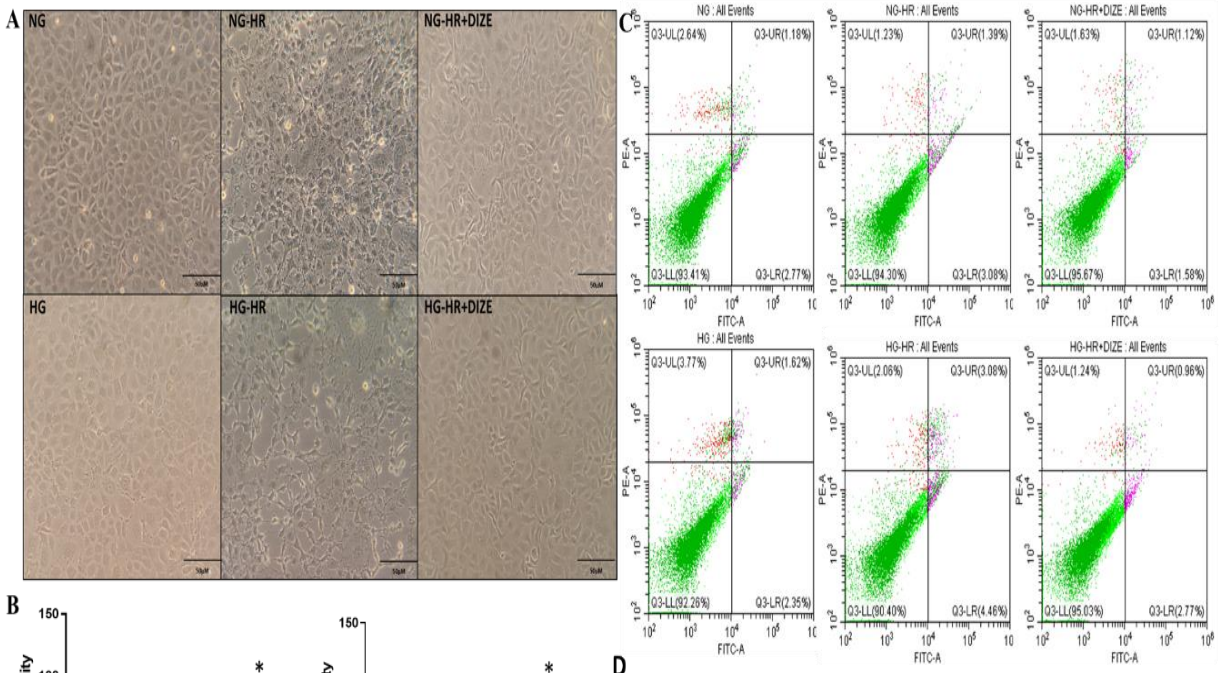


Figure 17: Effect of ACE2 activation on kidney histopathology. *A-B: representative images of IHC staining for MCP-1 and p-NF-kB in the outer cortex of the kidney were captured at 400x magnification with a 50 μm scale bar. At least 4-5 sections from each kidney (n = 6) were taken. D-E: Semi-quantitative analysis of all the images was done using ImageJ for calculating the DAB-positive area (indicates specific protein expressions). C: H and E staining were performed and the cortical region of kidney transverse sections was examined (original magnification 400× and scale bar- 50 μm). At least 4–5 images from each stained kidney section (n = 6) were observed by a blinded observer for tubular dilatation and injury (red arrow). F: The tubular dilatation and injury score was given from 0 to 3. All data are represented as mean ± SD. One-way ANOVA and Tukey's multiple comparison tests were used for statistical comparisons. (*) p < 0.05, vs NC; (^) p < 0.05 vs NC-I/R; ($) p < 0.05 vs DC; (#) p < 0.05 vs DC-I/R.*

5.2.3 Effect of ACE2 activation/inhibition on apoptosis and viability in NRK-52E cells

To determine the safe and effective doses of DIZE and MLN-4760 *in-vitro*, the XTT assay was performed using NRK52E cells. Different concentrations of DIZE ranging from 0 µM to 800 µM and MLN-4760 ranging from 1.25 µM to 80 µM were chosen (Fig. 18B).



D

Groups	NG	NG-HR	NG-HR+DIZE	HG	HG-H/R	HG-HR+DIZE
% of total apoptotic cells	3.95	4.47	2.78	3.97	7.54	3.73

Figure 18: Effect of DIZE and MLN-4760 on cell viability, cell morphology and apoptosis in NRK-52E cells. *A: The microscopic images of NRK52E cells were taken upon induction of HRI and treatment with the chosen dose. B: The NRK52E cells were treated for 24 h with different concentrations of diminazene aceturate (DIZE)- 0 µM to 800 µM and of MLN-4760 ranging from 1.25 µM to 80 µM followed by XTT assay were performed. C-D: The % apoptosis in NRK52E cells. Data are expressed as mean ± SD (n = 3). The one-way ANOVA followed by Tukey's multiple comparison test were used for statistical analysis, where (*) p < 0.05, vs NG; (^) p < 0.05 vs NG-H/R; ($) p < 0.05 vs HG; (#) p < 0.05 vs HG-H/R.*

Based on the XTT assay, doses of 100 μM for DIZE and 10 μM for MLN-4760 were selected for further studies (Fig. 18B). Kidney tubular epithelial cells are highly susceptible to apoptosis. Microscopically cells were observed to check the effect of HRI and treatment on cells morphology (Fig. 18A). HRI led to an alteration in cells morphology, such as cell shrinkage and condensation, loss of cell membrane, and change in shape (Fig. 18A). However, these changes were severe in presence of high glucose (HG-H/R). Interestingly, treatment with DIZE significantly restored the altered cell morphology (Fig. 18A). FACS imaging was performed to determine apoptosis after HRI and ACE2 inhibition/activation in NRK52E cells. FACS analysis revealed that HRI induced tubular apoptosis; however, apoptosis was significantly higher in the HG-H/R group (Fig. 18C, D) than in the NG-H/R group (Fig. 18C, D) ($p < 0.05$). Moreover, DIZE treatment effectively reduced the % of apoptotic cells ($p < 0.05$), indicating that ACE2 activation may have anti-apoptotic properties.

5.2.4 Effect of ACE2 activation on the expression of tubular proteins

We further determined the effects of ACE2 activation on tubular-specific protein expression. In both *in-vitro* and *in-vivo* tubular samples of AKI, the expression of Klotho (Fig. 19B, I) and ACE2 (Fig. 19C, J) significantly decreased under normal and hyperglycemic conditions ($p < 0.05$). However, Klotho expression was significantly lower in the HG-H/R group than in the NG-H/R group (Fig. 19B) ($p < 0.05$). Interestingly, the expression of Klotho and ACE2 was significantly restored after DIZE treatment both *in-vitro* and *in-vivo* ($p < 0.05$). Additionally, the levels of inflammatory proteins-p-NF-κB (Fig. 19E, L), p-IKK-α/β (Fig. 19F, M) and apoptotic proteins-cleaved-PARP (Fig. 19D, K) and C-cas-9 (Fig. 19G, N) were significantly increased in the HRI/IRI-induced AKI groups ($p < 0.05$), and their expression was further augmented in the presence of HG. After DIZE treatment, the expression of both inflammatory, as well as apoptotic proteins significantly reduced ($p < 0.05$). This suggests that ACE2 activation results in Klotho restoration and a decrease in inflammation and apoptosis, which eventually leads to recovery of kidney function in AKI.

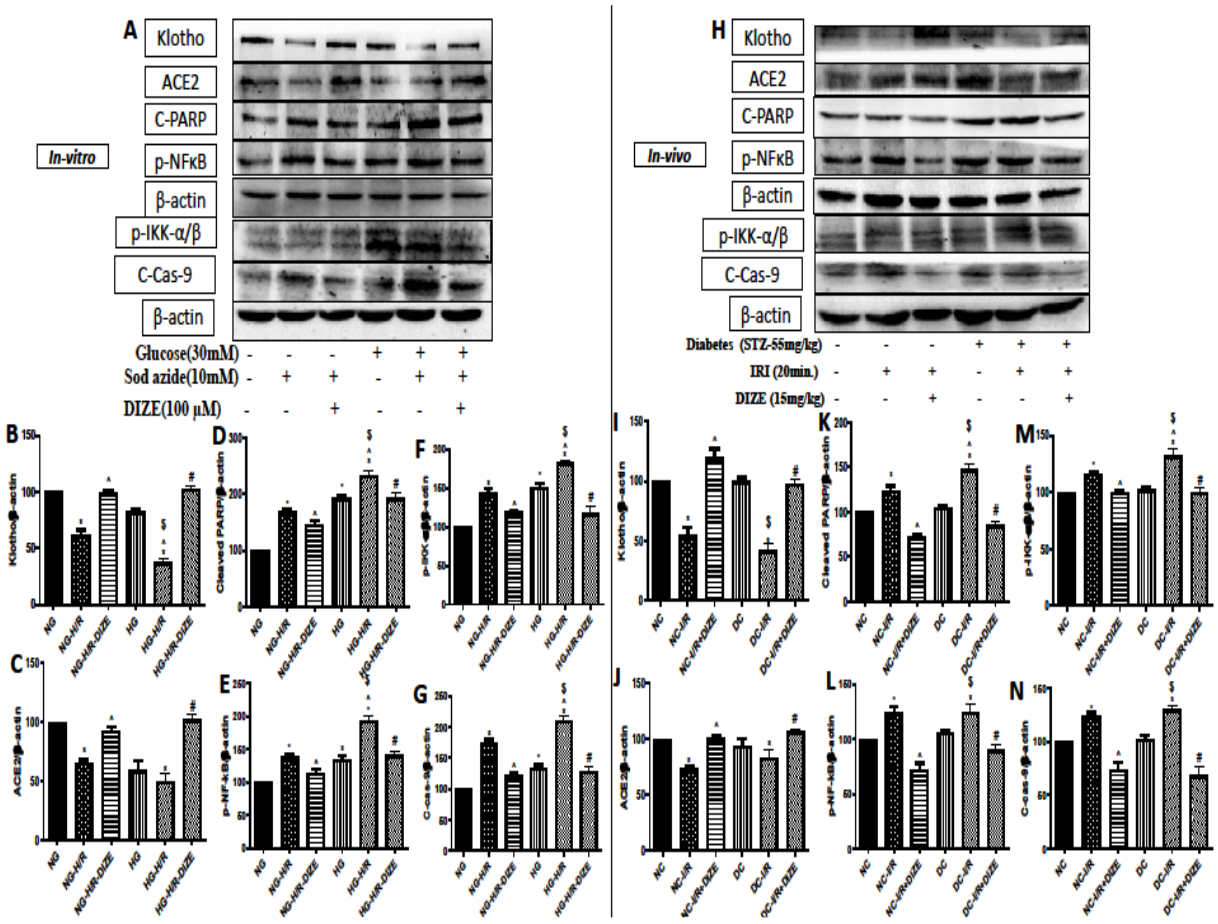


Figure 19: Effect of ACE2 activation on the expressions of tubular Klotho, ACE2, inflammatory and apoptotic proteins in-vitro and in-vivo. *A-N: Western blotting was performed using cell lysate of NRK52E (A) and isolated proximal tubular cells (H). The tubular expressions of proteins like Klotho (B, I), ACE2 (C, J), Cleaved PARP (D, K), p-NF-kB (E, L), p-IKK-α/β (F, M), and C-cas-9 (G, N) were checked (n =3). ll the values were represented as mean ± SD. One-way ANOVA and Tukey's multiple comparison tests were used for statistical comparisons. (*) p < 0.05, vs NG/NC; (^) p < 0.05 vs NG-H/R/NC-I/R; ($) p < 0.05 vs HG/DC; (#) p < 0.05 vs HG-H/R/DC-I/R.*

5.2.5 ACE2 regulates the Klotho expression in acute kidney injury under normal and hyperglycemic conditions

The effects of the non-conventional arm of the RAAS (ACE2-Ang-(1-7) axis) on AKI have not been explored. The role of ACE2 in Klotho regulation of AKI is unknown. To understand

this, we initially studied the effect of HRI and ACE2 inhibitors on Klotho and ACE2 expression in NRK52E cells under normal glucose conditions (Fig. 20A).

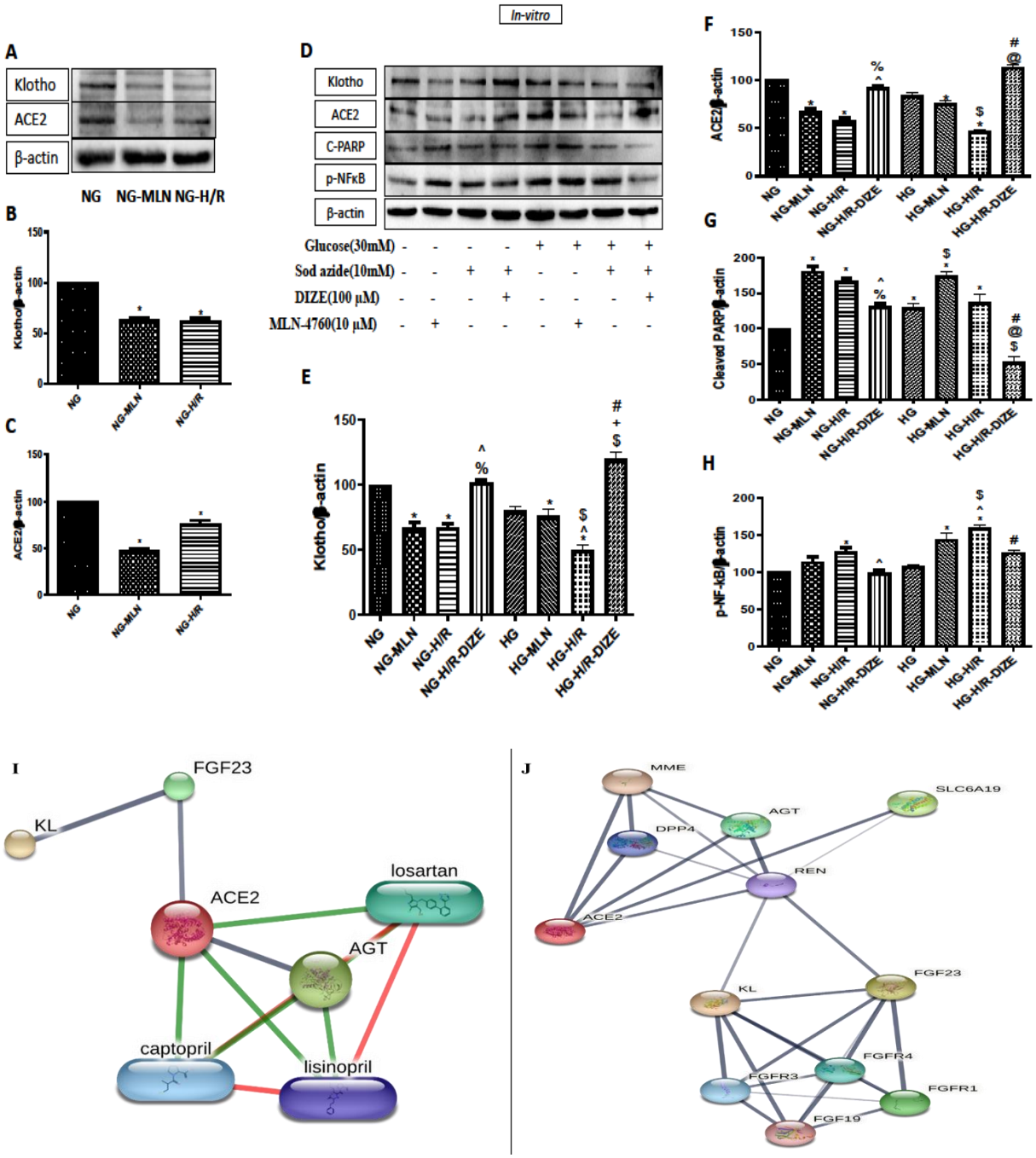


Figure 20: ACE2 regulates the Klotho expression in hypoxia-reperfusion injury-induced acute kidney injury under normal and high glucose conditions. *A-H: Proteins such as; Klotho (B, E), ACE2 (C, F), cleaved PARP (G) and p-NF-kB (H) in NRK52E cells were estimated (n=3). Densitometric analysis was employed for the immunoblots quantification.*

All experimental values were represented as mean ± SD. One-way ANOVA with Tukey's multiple comparison test was applied for statistical comparison. (*) p < 0.05, vs NG; (^) p < 0.05 vs NG-H/R; ($) p < 0.05 vs HG; (#) p < 0.05 vs HG-H/R. I-J: Klotho and ACE2 interaction analysis using in-silico tool.

In the HRI-as MLN-4760 treated group, the expression of Klotho (Fig. 20B) and ACE2 (Fig. 20C) was significantly reduced compared to that in the normal control groups (Fig. 20B, C) ($p < 0.05$). This indicated an interlink between ACE2 and Klotho in AKI. We further confirmed interaction between ACE2 and Klotho using in-silico sting and stitch tool (Fig. 20, I, J).

5.3 ER stress modulated klotho restoration: a prophylactic therapeutic strategy against acute kidney injury-diabetes comorbidity

5.3.1 Effect of ischemic AKI and ER stress modulation on Klotho and kidney function parameters in diabetic and nondiabetic rats

ER stress inhibitor TUDCA (400 mg/kg, *p.o.*) was administered for 3 days and 1 h before IRI.

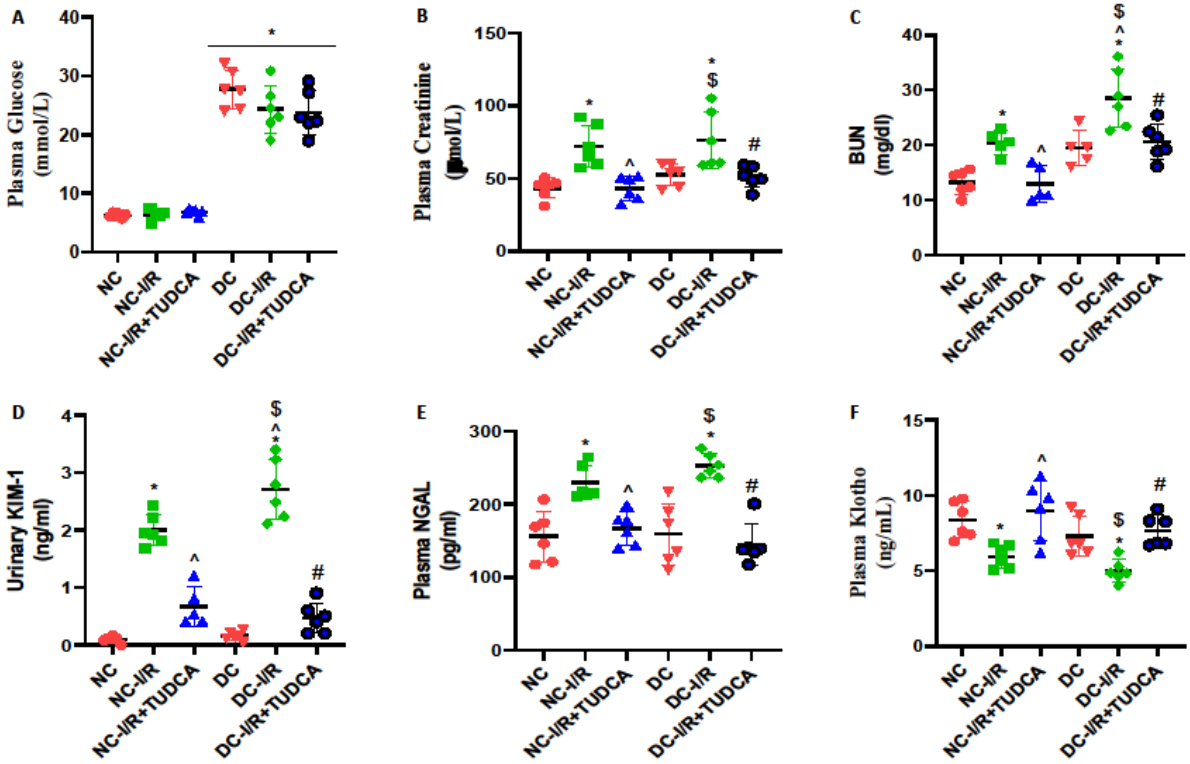


Figure 21: Effect of IRI and ER stress modulation on Klotho and kidney function

parameters. *A-F: The scattered plots represent the plasma glucose (A), plasma creatinine (B), BUN (C), urinary KIM-1 (D), plasma NGAL (E), and Klotho (F), where all experimental values are represented as mean ± SD (n=6). For statistical comparison the one-way ANOVA followed by Tukey's multiple comparison test was utilized, where (*) $p < 0.05$, vs NC; (^) $p < 0.05$ vs NC-I/R; ($) $p < 0.05$ vs DC; (#) $p < 0.05$ vs DC-I/R.*

Plasma glucose levels were significantly increased in the diabetic group, whereas TUDCA did not alter its level (Fig. 21A) ($p < 0.05$). After IRI, the plasma creatinine (Fig. 21B), BUN (Fig. 21C), NGAL (Fig. 21E), and urinary KIM-1 (Fig. 21D) levels were significantly elevated compared to those in the NC group ($p < 0.05$). Surprisingly, in the DC-I/R group, the levels of BUN and KIM-1 were higher than those in the NC-I/R group. The plasma Klotho level (Fig. 21F) was decreased after IRI, whereas in the presence of diabetes, it was reduced further. Fascinatingly, treatment with TUDCA significantly restored the altered levels of all these markers to normal (Fig. 21) ($p < 0.05$).

5.3.2 Effect of ER stress modulation and HRI on NRK52E cells viability and morphology

XTT assay was performed to determine the safe and effective doses of TUDCA and TUN using NRK52E cells (Fig. 22A, B).

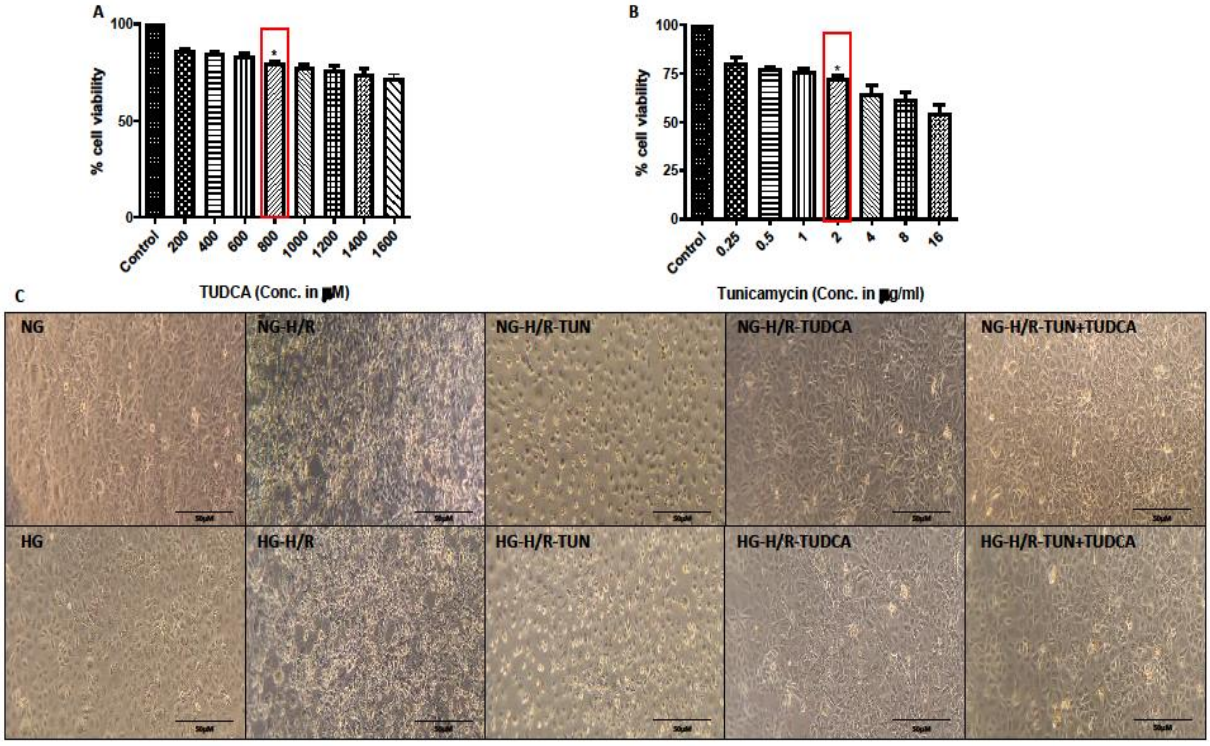
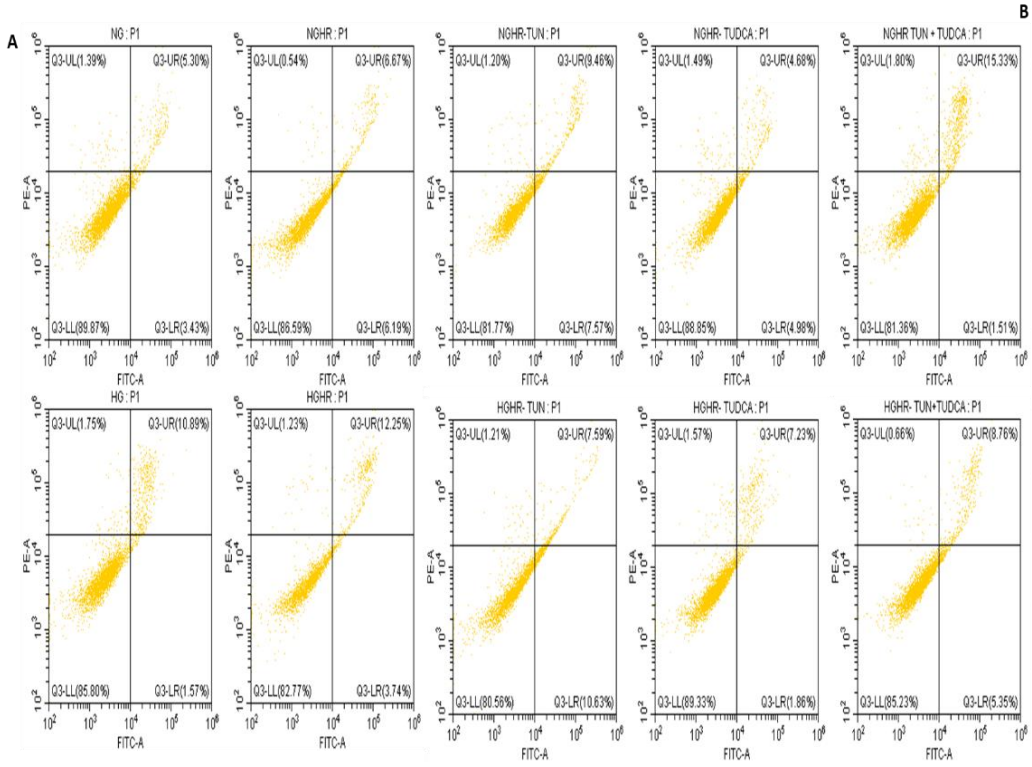


Figure 22: Effect of TUDCA and TUN on NRK52E cells viability and morphology. *A-B: The NRK52E cells were treated for 24 h with different concentrations of Tauroursodeoxycholic acid (TUDCA)- 0 µM to 1600 µM and of Tunicamycin (TUN) ranging from 0 µg/ml to 16 µg/ml followed by XTT assay were performed. C: The microscopic images of NRK52E cells were taken after the HRI and treatment with chosen doses of TUDCA and TUN under normal (5.5 mM) and high glucose (30 mM) conditions. Data are expressed as mean ± SD (n = 3). The one-way ANOVA followed by Tukey's multiple comparison test were used for statistical analysis.*

Based on the XTT analysis the doses of 800 µM for TUDCA (Fig. 22A) and 2 µg/ml for TUN (Fig. B) were chosen for further *in-vitro* studies. We also studied the effect of HRI and selected dosages of TUDCA and TUN on NRK52E cell's morphology (Fig. 22C).

5.3.3 Effect of HRI and ER stress modulation on apoptosis in NRK52E cells

HRI is known to induce cell apoptosis and is one of the key signaling mechanisms responsible for cell death in AKI development.



Groups	% apoptosis
NG	8.73
NGHR	12.86
NGHR-TUN	17.03
NGHR-TUDCA	9.66
NGHR-TUN+ TUDCA	16.84
HG	12.46
HGHR	15.99
HGHR-TUN	18.32
HGHR-TUDCA	9.09
HGHR-TUN+ TUDCA	14.11

Figure 23: Effect of HRI and ER stress modulation on % apoptosis in NRK52E cells.

To determine the % apoptosis, FACS imaging (using Annexin-V/FITC kit) was performed in HRI-induced and, TUDCA and TUN-treated NRK52E cells. The results from FACS analysis demonstrated that HRI (NGHR) led to tubular apoptosis induction wherein under high glucose conditions apoptosis was significantly increased (HGHR) (Fig. 23A, B). Importantly, this % was higher in TUN-treated cells indicating that ER stress induction further increases apoptosis (NGHR-TUN/HGHR-TUN). Moreover, in the TUDCA-treated groups (NGHR/HGHR-TUDCA) % of apoptotic cells was significantly reduced ($p < 0.05$) (Fig. 23A, B).

5.3.4 Effect of ischemia–reperfusion injury and ER stress inhibition on kidney histology of diabetic and non-diabetic rats

Hematoxylin and eosin (H&E) staining was performed to study the effect of IRI and TUDCA on the kidney morphology of diabetic and non-diabetic rats. IRI resulted in significant tubular injury and vasodilatation in the outer cortex region of the kidney when compared to the NC group (Fig. 24A, B) ($p < 0.05$). However, in the TUDCA-treated group, kidney morphology was unchanged. Importantly, under diabetic conditions, the kidney morphology additionally deteriorated and was successfully restored upon TUDCA treatment (Fig. 24A, B).

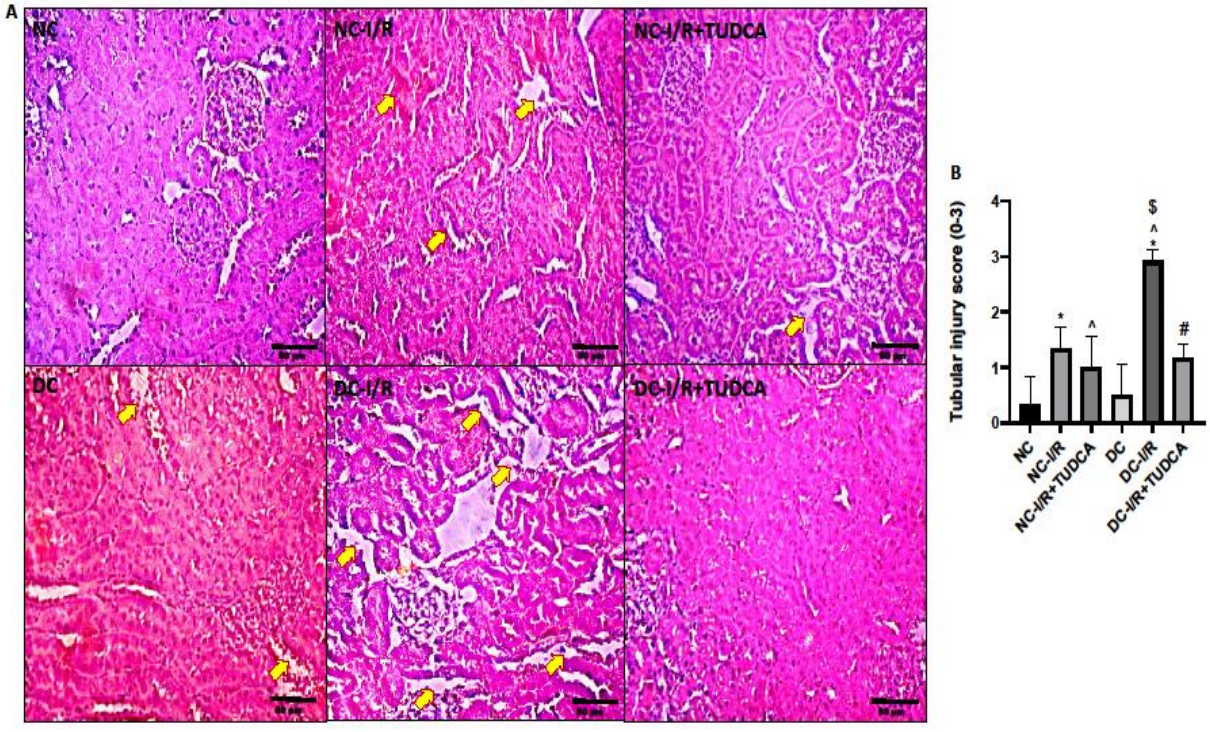


Figure 24: Effect of IRI and ER stress modulation on kidney morphology.

A: To study the kidney morphology, hematoxylin and eosin staining were performed and the cortical region of kidney transverse sections was observed (original magnification 400× and scale bar-50 µm). At least 4–5 images from each stained kidney section (n = 6) were examined by a blinded observer for tubular injury and dilatation (shown by a yellow arrow). B: The score for tubular dilatation and injury was given from 0 to 3. Data are expressed as mean ± SD. For the statistical analysis the one-way ANOVA followed by Tukey's multiple comparison test was used, where (*) p < 0.05, vs NC; (^) p < 0.05 vs NC-I/R; ($) p < 0.05 vs DC; (#) p < 0.05 vs DC-I/R.

5.3.5 Effect of ischemic AKI and ER stress modulation on the expression of kidney tubule-specific ER stress marker in diabetic and nondiabetic rats

Immunohistochemistry was performed to check the expression of p-PERK using kidney sections (Fig. 25). In the ischemic group (NC-I/R), the expression of p-PERK (Fig. 25A, B) was significantly increased, whereas under diabetic conditions (DC-I/R), their expression was further augmented. Interestingly, in the TUDCA-treated groups, the levels of p-PERK decreased significantly ($p < 0.05$).

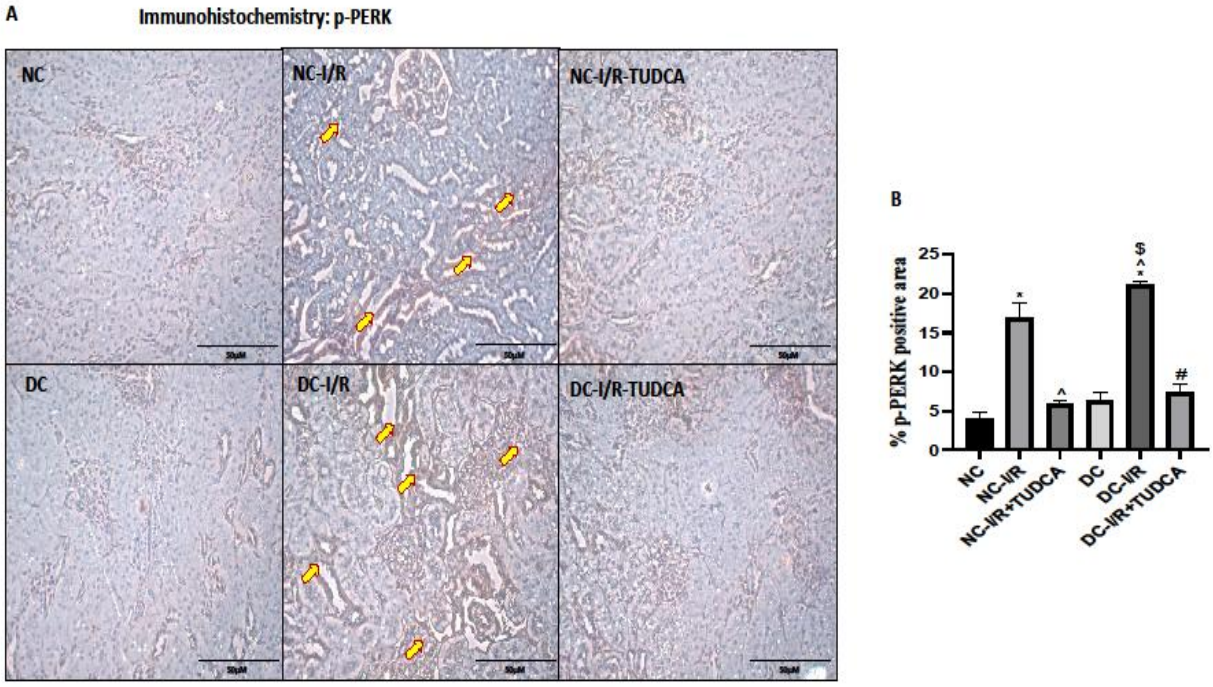


Figure 25: Effect of IRI and ER stress modulation on the expression of ER stress marker. *A: Representative image of IHC staining for p-PERK of the kidney (outer cortex region) were*

captured at 400× magnification with a 50 μM scale bar. At least 4–5 sections from each kidney (n = 6) were taken. B: Semi-quantitative analysis of the images was done using ImageJ software to calculate the DAB-positive area which indicates specific protein expressions (yellow arrow). Data are expressed as mean ± SD. For the statistical analysis the one-way ANOVA followed by Tukey's multiple comparison test was used, where (*) p < 0.05, vs NC; (^) p < 0.05 vs NC-I/R; ($) p < 0.05 vs DC; (#) p < 0.05 vs DC-I/R.

5.3.6 Effect of hypoxic/ischemic AKI and ER stress modulation on apoptotic and ER stress marker expression

To understand the role of ER stress and apoptosis in AKI and Klotho regulation, we performed a western blotting analysis using both *in-vitro* and *in-vivo* samples (Fig. 26).

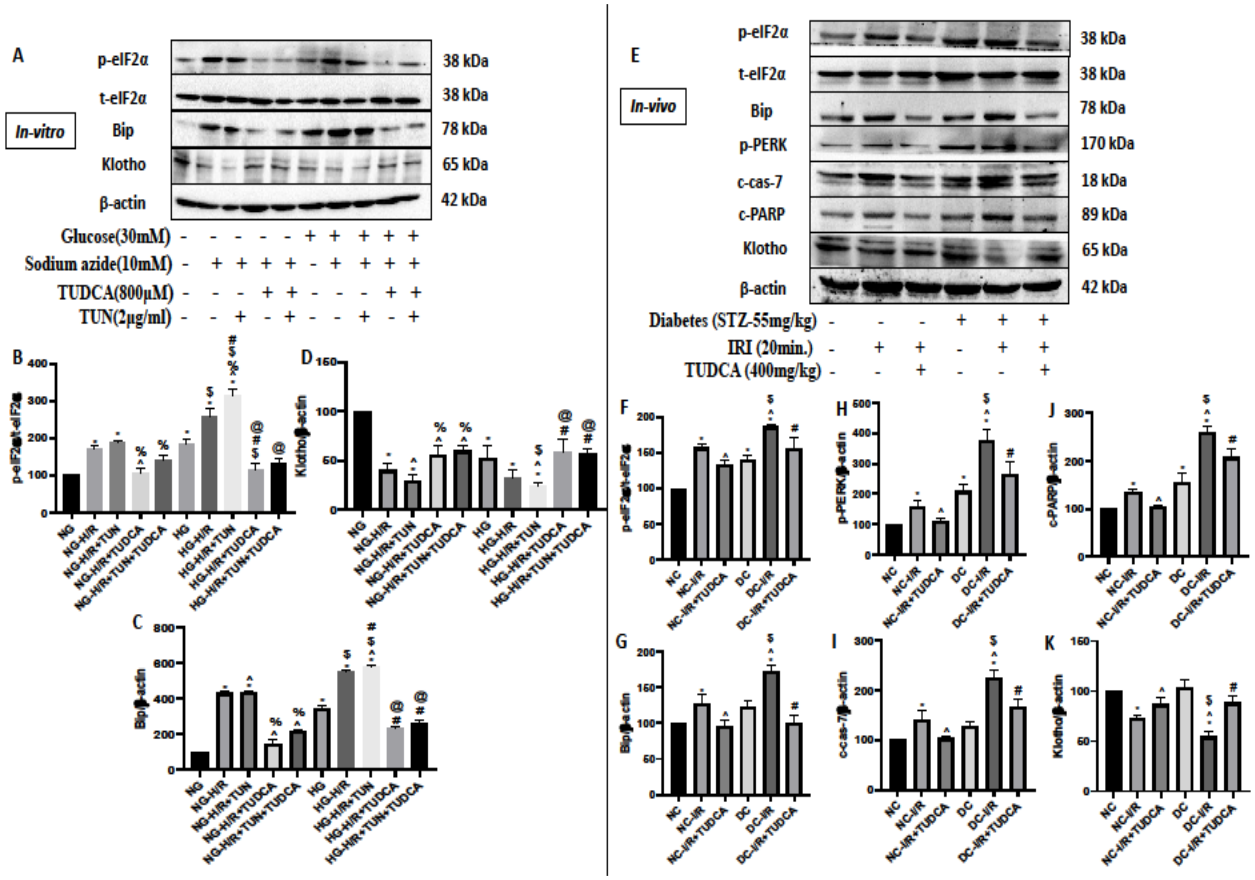


Figure 26: Effect of HRI/IRI and ER stress modulation on apoptotic and ER stress markers. *A-K: Western blotting was performed using cell lysate of NRK52E (A) and isolated proximal tubular cells (E). The tubular expressions of proteins like p-eIF2α (B, F), BiP (C,*

G), Klotho (D, K), p-PERK (H), c-Cas-7 (I), and c-PARP (J) were checked (n=3), where t-eIF2-α against p-eIF2α and β-actin against the other proteins were used as loading controls. Three independent experiments were conducted and all the values were represented as mean ± SD. For the statistical analysis, the one-way ANOVA followed by Tukey's multiple comparison tests was used. Where, (*) p < 0.05 vs NG/NC; (^) p < 0.05 vs NG-H/R/NC-I/R; ($) p < 0.05 vs HG/DC; (#) p < 0.05 vs HG-H/R/DC-I/R; (%) p < 0.05 vs NG-H/R+TUN; (@) p < 0.05 vs HG-H/R+TUN.

In-vitro, HRI increased the expression of ER stress markers such as p-eIF2α (Fig. 26B) and BiP (Fig. 26C) and decreased the Klotho level (Fig. 26D) ($p < 0.05$). When combined with treatment with the ER stress inducer TUN, BiP and p-eIF2α were further increased, and Klotho was significantly decreased (Fig. 26A). This suggests excessive ER stress and Klotho have an inverse relationship. Moreover, under hyperglycemic conditions, the levels of BiP and p-eIF2α were further increased, and Klotho levels were significantly decreased ($p < 0.05$). In parallel, *in-vivo* studies also showed similar results. IRI led to a significant rise in apoptotic (Fig. 26I, J) and ER stress markers (Fig. 26F, G, H) and a decrease in Klotho expression (Fig. 26K) ($p < 0.05$). Moreover, under diabetic conditions (DC-I/R), apoptotic and ER stress marker expression was further elevated, and Klotho expression was significantly decreased compared to the nondiabetic groups (NC-I/R) (Fig. 26E) ($p < 0.05$).

6. Discussion

AKI is an abrupt decline or loss of kidney functions (PA Cass et al., and Nisha et al., 2020). Risk, Injury, and Failure, and Loss (RIFLE) criteria apprise that, AKI is not just renal failure but also includes the necessity of renal replacement therapy (JPGaH Liapis, 2020). From the clinical and prognostic perspective, AKI is detrimental health complications and remains the imperative fundamental problem with a consistent rise in morbidity and mortality rates across the globe (J.J Chen et al., 2021). Moreover, morbidity and mortality rate of AKI is very high in diabetic patients, which stipulate that hyperglycemia may be an independent risk factor for AKI (A. Advani, 2020). But the underlying mechanism involved in the same is still elusive. However, Klotho is a crucial reno-protective protein that manifests an inverse relationship with AKI progression (HJ Oh H Oh, et al., 2019). Klotho has three different types including, α-Klotho, β-Klotho and γ-Klotho where its expression and function is distinct from organ to organ. Here, we focused on α-Klotho only (mentioned as a Klotho) which is abundantly expressed by proximal convoluted tubules of the kidney. Klotho is being explored as a diagnostic biomarker and therapeutic target for various forms of kidney disease (O Zou, et al., 2018 and JA. Neyra et al., 2021). In kidney disease severe systemic and renal Klotho deficiency is reported. This notion is also supported by clinical reports. However, its endogenous restoration and exogenous supplementation has shown renoprotective effects in various forms of acute and chronic kidney disease such as kidney fibrosis, DKD, DN, toxin induced kidney injury, etc. Also, in diabetic and cardiovascular conditions decreased Klotho level is reported. Considering this extensive Klotho loss could be a probable factor for increased prevalence of AKI incidences in hyperglycemic patients, which is not explored yet. Additionally, the underlying molecular mechanisms that plays a significant role in Klotho regulation during the progression of AKI in the presence or absence of hyperglycemia is inadequately known (J. Xia, W. Cao, 2021). Hence, through this study, we evaluated the therapeutic potential and also studied the epigenetic and non-epigenetic mechanisms of Klotho regulation against the ischemic AKI under diabetic and non-diabetic conditions.

6.1 Epigenetic restoration of endogenous Klotho expression alleviates acute kidney injury-diabetes comorbidity

Epigenetics is heritable changes in DNA and associated proteins except for mutations in genes or alterations in the original DNA sequence. Particularly, alterations in acetylation/deacetylation status of histone and non-histone proteins by kidney-specific histone deacetylases (HDACs 1,2,3,6 and 8) showed an alliance with Klotho modulation and kidney disease progression (W Lin, et al., 2017 and X.H. Yang, at al., 2021). However, their impact on Klotho regulation and underlying pathological mechanisms involved in AKI-hyperglycemia comorbidity is unknown. We extensively reviewed the epigenetic and non-epigenetic mechanisms of Klotho regulation in kidney diseases and found that the kidney-specific histone deacetylases remarkably downregulate the endogenous Klotho level and promotes kidney disease. This indicates the importance of studying the Klotho regulation in AKI. Thus, it becomes imperative to explore the effect of hyperglycemia on HDACs expression, H3 protein levels and modulation of endogenous Klotho in AKI. Furthermore, determinants of Klotho downregulation and signaling pathways associated with it are a completely unexplored area of the research in AKI-hyperglycemic comorbidity.

In this study, we firstly explored the impact of hyperglycemia on AKI progression followed by the role of kidney-specific histone deacetylases (HDACs 1,2,3 and 6) on Klotho regulation. We also studied the AKI interlinked pathological signaling mechanisms such as apoptosis, inflammation, and their association with Klotho in AKI-hyperglycemic comorbidity. To achieve this, we performed the bilateral ischemia-reperfusion injury, a surgery model in type 1 diabetic and non-diabetic Wistar rats and the chemical hypoxia reperfusion injury model of AKI in NRK52E cells in presence of normal glucose (5.5 mM) and high glucose (30 nM). Our study discovered the role of HDACs in the endogenous Klotho regulation in AKI-hyperglycemic comorbidity. The present study also checked the protein levels of histones - H3K9 and H3K27 and their association with HDACs and Klotho expression. We hypothesized that epigenetic modulation of Klotho using TSA a Pan-HDAC inhibitor can stop endogenous Klotho loss, which eventually may halt the AKI progression under hyperglycemic and non-hyperglycemic conditions. To address this, we conducted both *in-vivo* and *in-vitro* experiments. Key findings of our study comprise of i) hyperglycemia exaggerates the IRI/HRI induced AKI; ii) AKI is the state of systemic and renal Klotho

deficiency where hyperglycemia leads to additional Klotho insufficiency; iii) IRI/HRI increases tubular expressions of kidney-specific HDACs (1,2,3 and 6) and, decreases H3K27Ac and H3K9Ac protein level in which hyperglycemia further altered their expressions; iv) TSA produced reno-protection via HDACs inhibition and endogenous Klotho restoration, and v) Klotho is essential for TSA mediated reno-protection in AKI.

The BUN, plasma creatinine, KIM-1, neutrophil-gelatinase-associated lipocalin (NGAL), and Klotho are some of the potential biomarkers of AKI (J.L. Alge, and J.M. Arthur, 2015). The level of these biomarkers indicates the severity of AKI. In the presence of a hyperglycemic condition, these biomarkers further go high. In the present study, IRI-induced AKI showed that BUN, plasma creatinine and KIM-1 level was increased and Klotho was decreased (Fig. 10). Interestingly, we found that hyperglycemia significantly added to the damage caused by AKI observed both biochemically and morphologically. This indicates that hyperglycemia plays an important role in AKI aggravation. We also reported a similar finding in our previous studies (N Sharma, et al., 2020). However, TSA, a Pan-HDAC inhibitor successfully restored the expression of these biomarkers. Apart from these biochemical changes, IRI altered the morphology of the rat kidney. Particularly, proximal tubular cells are highly susceptible for IRI induced AKI, characterized by loss of brush border, tubular dilatation and necrosis (L.M.S. Gerhardt, and A.P. McMahon, 2021). In the present study, IRI/HRI resultant into morphological changes in renal proximal tubular cells was restored by TSA a Pan-HDACs inhibitor (Fig. 11). The aberrant increase in expressions of histone deacetylase contributes to the progression of AKI via controlling the different cellular pathological processes such as inflammation, apoptosis, and renal fibrosis (F. Chen, et al, 2021). HDACs usually do the chromatin modification1s by forming the complex with transcription factors or changing the acetylation status of histones (S Cheng, et al., 2021). Recent studies have shown that inhibition of HDACs activation using HDACs inhibitors like vorinostat, romidepsin, TMP195, ameliorates AKI. To look into the role of HDACs and their effects on Klotho regulation in AKI-hyperglycemia comorbidity we further conducted molecular studies. Here we found that HRI/IRI significantly increased the expression of kidney-specific HDAC-1,2,3 and 6, and in contrast repressed the protein level of Klotho, H3K9Ac and H3K27Ac *in-vitro* and *in-vivo* (Fig. 12). In hyperglycemic IRI/HRI groups, the expression of HDACs was further elevated and, H3K9Ac and H3K27Ac histones were significantly downregulated when compared to

normal control and non-hyperglycemic IRI groups. These findings suggest that epigenetic modulations like aberrant HDACS expression and decreased levels of histones H3K9Ac and H3K27Ac worsen hyperglycemia exaggerated AKI via Klotho loss. However, TSA a Pan-HDAC inhibitor produce reno-protection via endogenous Klotho restoration in AKI-hyperglycemia comorbidity (Fig. 12).

Multiple underlying mechanisms are involved in the pathogenesis of AKI and among those, apoptosis is the most predominant mechanism that leads to structural and functional changes in kidney cells (A. Havasi, and S.C. Borkan, 2011). IRI/HRI model is already known for inducing apoptosis in kidney cells (W Chen, et al., 2019). Also, another study identified that Klotho reduces the expression of apoptotic proteins and thus may possess anti-apoptotic properties (M.C. Panesso, et al., 2014). However, in presence of hyperglycemia, an association between Klotho and apoptosis is unknown. Additionally, the effect of histone deacetylase inhibition on Klotho and apoptosis remains unexplored. In the present study, in presence of hyperglycemia, IRI/HRI resultant into apoptosis and Klotho downregulation in kidney indicated by an increase in percentile of apoptotic NRK-52E cells *in-vitro* (shown by flow cytometry) along with an increase in expression of apoptotic proteins such as cleaved-PARP, cleaved-caspase 3 and cleaved-caspase 9 both *in-vitro* and *in-vivo* (Fig. 13). Here also in presence of hyperglycemia, apoptosis was inflated as indicated by a significant increase in, percentile of apoptosis *in-vitro* and *in-vivo* compared to control and non-hyperglycemic IRI/HRI groups. However, TSA reduced the apoptotic cells death and expressions of these apoptotic proteins along with Klotho restoration *in-vitro* and *in-vivo* (Fig. 13, 14). It demonstrates that TSA mediated HDACs inhibition shows anti-apoptotic activity via partial restoration of endogenous Klotho in AKI under hyperglycemia.

Inflammatory pathways, particularly NF-κB and associated transcriptional factors are paramount to AKI progression and thus considered as the main target for anti-inflammatory based therapies (T Liu, et al., 2017). It is seen that hyperglycemia, as well as AKI, triggers the NF-κB activation via distinct pathways (Y. Li, and K. Ren, 2020). Interestingly Klotho also shows the association with NF-κB signaling in AKI (H Li, et al., 2020). However, the above findings are not explored in hyperglycemia aggravated AKI. Our results showed that IRI/HRI activated the NF-kB signaling as evidenced by an increase in expression of MCP-1, p-IKK-α/β, p-NF-κB proteins level and TNF-α mRNA expressions (Fig. 14). Compared to

the control group, IRI/HRI with hyperglycemia significantly increased the expression of aforesaid proteins. In contrast, the protein and mRNA expression of Klotho was significantly reduced. HDACs inhibition instigated Klotho restoration and inhibition of the NF-κB signaling. Thus, we conclude that hyperglycemia may aggravate the AKI by further Klotho downregulation and activation of NF-κB signaling.

The findings of our study confirmed that diabetes is an independent risk factor responsible for AKI exaggeration. The HDACs inhibition leads to endogenous Klotho restoration and thus AKI amelioration both in-vitro and in-vivo (Fig 27). However, to further confirm that the reno-protection observed upon HDACs inhibition was because of Klotho restoration, we conducted a Klotho siRNA transfection in NRK52E cells. Surprisingly, in the Klotho knockdown group, the expression of inflammatory and apoptotic proteins remained higher even after the HDACs inhibition (Fig. 15). However, Klotho siRNA transfection did not alter the status of HDACs proteins. ***It indicates that Klotho is one of the key connecting link between diabetes and AKI, and the HDACs inhibition mediated reno-protection in AKI-diabetes comorbidity.***

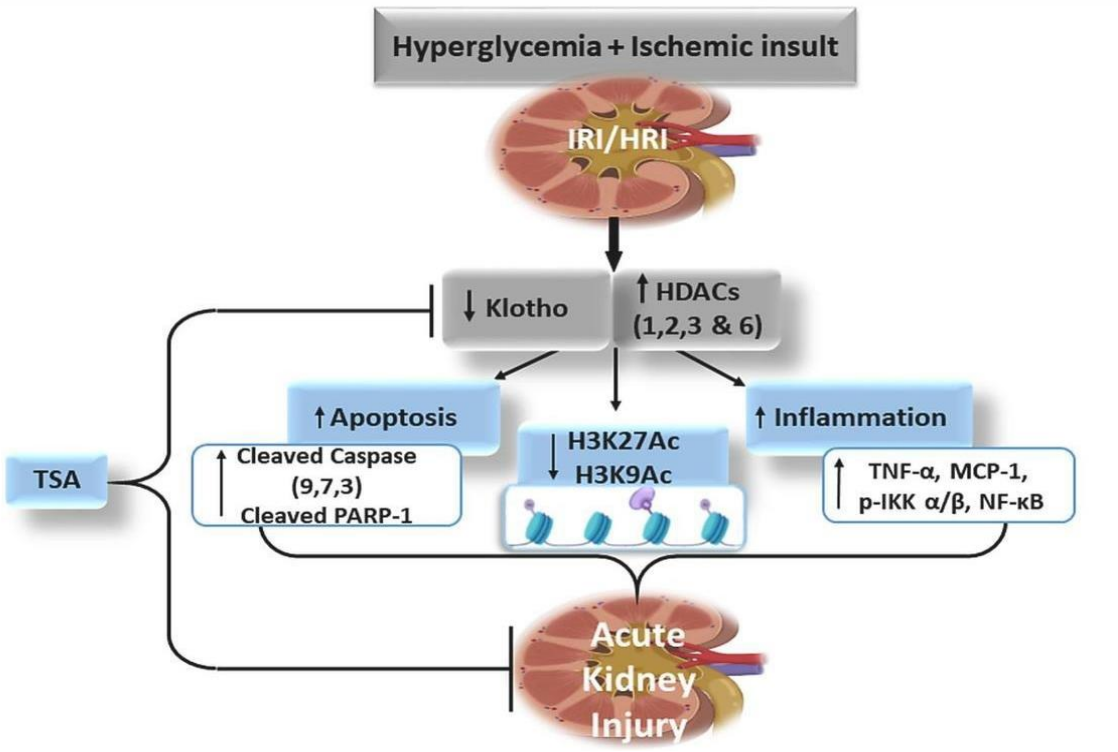


Figure 27: Overview of Klotho and epigenetics in acute kidney injury-diabetes comorbidity

6.2 Klotho restoration via ACE2 activation: A potential therapeutic strategy against acute kidney injury-diabetes comorbidity

The renin-angiotensin-aldosterone system (RAAS) is composed of two arms. The conventional arm of RAAS is known to trigger apoptosis, inflammation, oxidative stress, and fibrotic signaling events, eventually promoting AKI progression (N Sharma, et al., 2019 and E. Dudoignon, et al., 2019). However, the role of nonconventional RAAS in AKI progression has yet to be explored. For example, in a mouse model of ischemic AKI, the mRNA and protein levels of renal ACE2 decreased compared to those in the normal control groups, indicating the importance of ACE2 regulation in AKI (F. Fang, et al., 2013). Basic research has delineated the protective effects of non-conventional RAAS in AKI, but the underlying mechanisms involved are still elusive. Remarkably, recent preclinical studies have observed that non-conventional RAAS upregulates and activates Klotho in endothelial cells and related vascular complications, demonstrating significant improvements in their functional and structural characteristics (A. Romero, et al., 2019). ***However, the role of non-conventional RAAS in Klotho regulation of AKI is still being determined.*** Recent studies have shown that ACE2 deficiency is present in AKI, where its activation shows AKI amelioration (M. Malek, and M. Nematbakhsh, 2014). However, the molecular mechanisms underlying its reno-protective effects remain unclear. Importantly, the effect of ACE or Ang-II on Klotho regulation is known however the effect/role of ACE2 in Klotho regulation in AKI yet unknown. ***Thus, we hypothesized that during the AKI progression under normal and diabetic conditions, the reduced endogenous Klotho level can be restored via ACE2 activation (using an ACE2 activator i.e. DIZE).*** Henceforth, through this study we explored the effects of ACE2 activation on Klotho expression, the underlying pathomechanisms and signaling pathways that are involved in IRI/HRI induced AKI under diabetic and non-diabetic conditions. In agreement with previous findings, our biochemical results suggested that diabetes exacerbates AKI. In AKI, an increase in the expression of RAAS components, such as Ang-II, ACE, or AT1R, is well known. Also, suppression of the ACE2/Ang-(1-7)/MasR axis initiates pathological processes such as inflammation, fibrosis, and apoptosis (M. Legrand, and M.P. Bokoch, 2021). However, the relationship between RAAS and Klotho in IRI-induced AKI requires further investigation. Our biochemical analysis suggested that IRI results in a decrease in renal ACE2 and plasma Ang-(1-7) levels along with a significant

reduction in plasma Klotho levels, where diabetes further exacerbates the condition (Fig. 16). This suggests that IRI-induced AKI results in the repression of ACE2-Ang-(1-7) as well as Klotho. ACE2 enzymatically regulates the synthesis of inactive Ang-(1-9) from Ang-I and converts Ang-II into Ang-(1-7); thus the systemic and tissue levels of Ang-(1-7) are ACE2 dependent (L.F. Alawi, et al., 2021). Interestingly, ACE2 activation efficiently augmented renal ACE2 levels, followed by plasma Ang-(1-7) and Klotho levels. These findings signify that the ACE2-Ang-(1-7) axis may play a role in Klotho regulation and, thus, renoprotection in AKI. In support of this notion, another study showed that Klotho activation by the Ang-(1-7)/MasR axis protected endothelial cell senescence (A. romero, et al., 2019). To explore this at the molecular level, we also examined the proximal tubule-specific expression of ACE2 and Klotho. IRI accounts for a significant reduction in tubular ACE2 and Klotho levels in conjunction with the activation of pathomechanisms such as inflammation and apoptosis (Fig. 18, Fig. 19). ACE2 is known to inhibit inflammation and apoptosis in AKI, but the underlying molecular mechanisms remain unclear. Hu et al. recently showed that Klotho suppresses apoptosis in hypoxia and ischemia-reperfusion injury models (J. Hu, et al., 2021). Klotho has also been reported to have anti-inflammatory activities mediated by inhibition of the translocation and activation of NF-κB, as well as by inhibition of the transcription of pro-inflammatory genes (P. Buendía, et al., 2016). In this study, DIZE treatment restored tubular expression of Klotho, followed by the inhibition of inflammation and apoptosis (Fig. 18, Fig. 19). Hence, it can be said that the inhibition of inflammation and apoptosis that occurs after DIZE treatment is mediated via Klotho restoration. These outcomes also indicate that ACE2 may upregulate Klotho expression, which eventually leads to renoprotection in AKI. To support this notion, we conducted an in-silico study which demonstrated that, ACE2 and Klotho interact via fibroblast growth factor (FGF)-23 and renin signaling. Interestingly, FGF-23 and renin is highly expressed in kidney where Klotho shows the inverse relationship with them (M Christow, et al, 2019 and M. Freundlich, et al., 2021). Similarly, increased level of Ang-II and FGF-23 led to ACE2 downregulation. However, Ang-II inhibition results in decrease in FGF-23 level and increase in ACE2 expression (I. Böckmann, et al., 2019). Our previous findings already revealed the mechanisms and roles of different RAAS components during AKI and diabetic kidney disease: effect of ACE2 activation on different RAAS components like, Ang-(1-7), Ang-II, ACE, etc. (S. Nisha, et al., 2020 and SK Goru, et al.,

2017). However, association between these RAAS components and Klotho was unexplored. Cumulatively, these findings suggest the direct or indirect interaction between Klotho and ACE2 via FGF-23 and renin modulation. In the present study, we did not evaluate the FGF-23 and renin expression to verify this finding, that is the potential limitation of the study.

However, to further confirm this hypothesis, we exposed NRK52E cells to MLN-4760, a specific ACE2 inhibitor. HRI led to a significant reduction in ACE2 and Klotho levels (Fig. 20). Surprisingly, ACE2 inhibition also resulted in a significant decrease in Klotho levels (Fig. 20B and E). In advance, the decrease in Klotho level was associated with an increase in the level of the renal inflammatory marker p-NF-kB and apoptotic marker cleaved-PARP (Fig. 18, 19). In support of the above-discussed studies, our results also suggest that ACE2 plays a role in renal inflammatory and apoptotic signaling (SK Goru, et al., 2017). Interestingly, ACE2 activation restored Klotho, p-NF-κB, and cleaved-PARP. ***Thus, the overall results suggest that ACE2 may upregulate systemic and tubular Klotho expression, thereby demonstrating renoprotection against AKI (Fig. 28).***

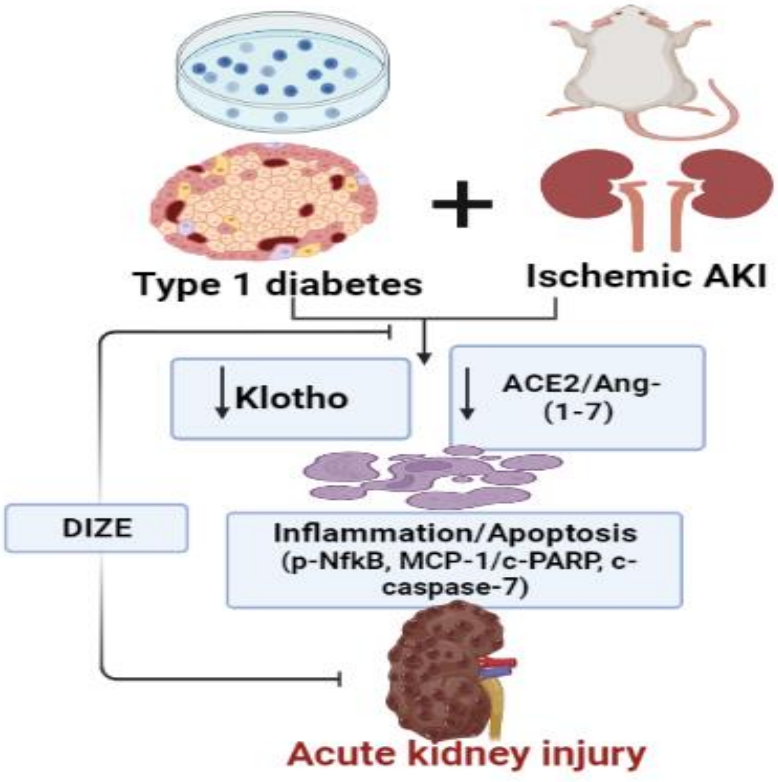


Figure 28: Overview of effect of ACE2 activation on Klotho regulation in acute kidney injury under diabetic conditions.

6.3 ER stress modulated klotho restoration: a prophylactic therapeutic strategy against acute kidney injury-diabetes comorbidity

The endoplasmic reticulum is the primary site for protein synthesis, folding, processing, and transport and regulates proteostasis (H. Sankrityayan, et al, 2019). ER is also responsible for lipid synthesis and storage, $Ca2^{+}$ release, and insulin signaling. However, perturbations in cells or an increased burden on ER results in the disruption of ER proteostasis. Recent studies have also demonstrated that IRI induces excessive or persistent ER stress in tubular cells of the kidney during AKI (M Yan, et al., 2018). Interestingly, reports have suggested a causal relationship between Klotho and ER Stress. However, the effect of ER stress and its inhibition on Klotho regulation or vice versa in IRI-induced AKI under diabetic/hyperglycemic conditions remains a matter of investigation.

Herein, we hypothesized that ER stress might modulate Klotho expression, which eventually plays an essential role in the pathogenesis of IRI-induced AKI in the presence of type 1 diabetes/hyperglycemia. Hence, using an ER stress inhibitor- TUDCA we could restore endogenous Klotho expression and ameliorate ischemic AKI under hyperglycemic conditions. To achieve this, we conducted a set of experiments using *in-vitro* and *in-vivo* models of ischemic AKI perse and under hyperglycemic conditions. In parallel with our previous findings, biochemical results demonstrated that hyperglycemia exaggerates ischemic AKI, as the levels of BUN and KIM-1 were significantly high and Klotho levels were significantly low in the diabetic IRI group (Fig. 21). Interestingly, TUDCA, an ER stress inhibitor, restored the altered levels of these markers, indicating the potential role of ER stress in AKI pathogenesis and Klotho regulation.

Perturbation of tubular cells in IRI-induced AKI activates the UPR as an adaptive mechanism. However, persistent UPR activation results in the stimulation of the apoptotic pathway to eliminate misfolded proteins or damaged cells (R Lurlaro, et al., 2016). Our *in-vitro* data show that upon exposure of NRK52E cells to HRI and the ER stress inducer TUN, the morphological characteristics of the cells were changed (Fig. 23). The reason could be the activation of the apoptotic pathway by excessive ER stress. Similarly, the histological analysis of the kidney clearly shows increased tubular dilatation and injury in the cortex region of the kidney in the IRI groups, wherein in the presence of diabetes, the threshold of the injury was greater (Fig. 24A, B). Fascinatingly, TUDCA treatment inhibited kidney injury. These

findings suggest that ER stress has a role in kidney damage and may initiate other pathological processes eventually leading to necroptosis. Our IHC results demonstrated increased expression of both p-PERK (ER stress marker) in the cortex region of the kidney after IRI (Fig. 25). Here, also, in the presence of hyperglycemia, the expression levels of these markers were significantly high. Moreover, the TUDCA-treated groups showed negligible expression of these markers. However, the downstream effect of these elevated markers is poorly understood.

Excessive ER stress as a result of IRI results in the persistent activation of the UPR to repair and normalize proteostasis. Moreover, over time, it activates apoptosis to degrade and remove misfolded proteins or damaged organelles. To evaluate this phenomenon in hypoxic/ischemic AKI under diabetic and non-diabetic conditions, we performed FACS analysis in NRK52E cells and also estimated the expression of other potential ER stress markers, such as Bip, p-PERK, and p-eIF2-alpha, and apoptotic markers, such as cleaved PARP and cleaved-caspase-7, by immunoblotting (Fig. 25 and Fig. 26). Our results revealed that post-HRI/IRI, there was an increase in ER stress and apoptosis, wherein diabetes has exaggerated these signaling pathways. Interestingly, TUDCA efficiently downregulated the expression of both ER stress and apoptotic markers. This suggests that ER stress is a shared pathomechanism for AKI and diabetes, which further activate apoptosis.

Apart from ER stress inhibition, TUDCA may play a role in Klotho regulation. ER stress is a shared pathomechanism responsible for the progression of AKI and diabetes; likewise, Klotho is a common protective factor for AKI and type 1 diabetes alone (A Zubkiewicz-Kucharska, et al., 2021 and J Flotyńska, et al., 2018). Thus, in AKI-diabetes comorbidity, ER stress inhibition and Klotho upregulation are possible. Hence, to confirm this, we estimated Klotho expression both *in-vitro* and *in-vivo*. Klotho expression was significantly decreased after AKI both *in-vitro* and *in-vivo* (Fig. 26D, K). We further exposed HRI-induced NRK52E cells to the ER stress inducer TUN to determine the effect of excessive ER stress on Klotho**.** ***Remarkably, excessive ER stress resulted in a significant decrease in Klotho (Fig. 26D), indicating a strong association between ER stress and Klotho. Surprisingly, under hyperglycemic conditions, the expression of Klotho was further decreased (Fig. 29).***

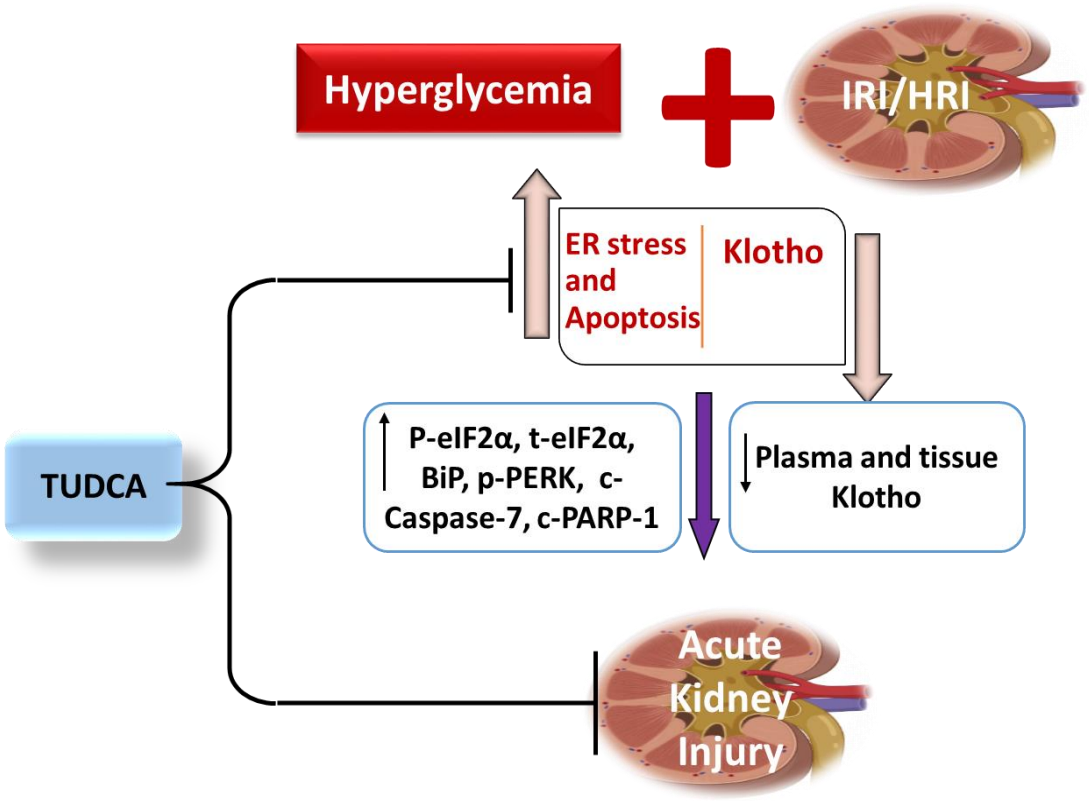


Figure 29: Overview of effect of ER stress and inhibition on Klotho regulation in acute kidney injury under diabetic conditions.

Overall outcomes of the study suggest that ischemic AKI is the state of Klotho deficiency wherein type 1 diabetes further downregulates Klotho and exaggerated AKI. Endogenous level of Klotho can be restored by targeting epigenetic (via HDAC inhibition and non-epigenetic mechanisms (via ACE2 activation and ER stress inhibition) in ischemic AKI. An endogenous restoration of Klotho by targeting these mechanisms ameliorates AKI under type 1 diabetic as well as non-diabetic conditions. Hence, Klotho can be considered as novel and promising therapeutic target against the ischemic AKI in presence or absence of type 1 diabetes. However, to further validate Klotho's therapeutic potential, more preclinical and small scale clinical studies should be conducted.

7. Conclusion

- Role of diabetes and epigenetics in Klotho regulation and AKI development was the unexplored area of research. Through this study we revealed that IRI/HRI induced AKI showed marked elevation in kidney specific HDACs expressions along with activation of apoptosis, inflammation cascade and reduction in Klotho level. Moreover, in presence of diabetes severity of AKI was significantly enhanced, indicating that diabetes is an independent risk factor for AKI. However, HDAC inhibition using TSA, re-established the endogenous Klotho loss, inhibited the apoptosis and inflammation which eventually attenuated the AKI. Hence, in future, targeting epigenetic mechanism for endogenous Klotho restoration may prove novel therapeutic strategy against AKI-diabetes comorbid patients. However, further pre-clinical and clinical investigations are needed to confirm these results in AKI-diabetes comorbid patients.
- Renin-angiotensin system (RAAS) has diverse role in the pathogenesis of AKI. However, non-conventional arm of RAAS and its effects on Klotho regulation in the kidney disease is inadequately known. We found that ACE2 is a protective component of the RAAS, and its inhibition/deficiency leads to Klotho downregulation and triggers inflammation and apoptosis during the course of AKI. However, treatment with diminazene aceturate, increased the ACE2 and Ang-(1-7) levels, restored endogenous Klotho loss, and showed significant reno-protection in AKI. The study outcomes also suggest that Klotho may act as an intermediate for ACE2-mediated reno-protective effects in IRI/HRI-induced AKI under normal and high glucose conditions. Thus, in the future, targeting renal ACE2-Ang-(1-7)-Klotho axis by either increasing their endogenous levels using any therapy or exogenous administration could prove a promising therapeutic strategy against ischemic AKI. However, this is the first preclinical study to report this novel finding and hence more preclinical and clinical studies are required to be conducted to verify its therapeutic potential.
- Endoplasmic reticulum (ER) plays important role in proteins synthesis and folding. In our study IRI/HRI led to increase in ER stress, as manifested by increased level of ER stress and apoptotic markers. Subsequently, the significant reduction in Klotho level was observed, which was further decreased in presence of diabetes. Interestingly, TUDCA-mediated ER stress inhibition restored Klotho loss and ameliorated AKI. The outcome of

this study suggests that ER stress and Klotho regulation are interlinked factors in AKI-diabetes comorbidity, and targeting them may provide a novel therapeutic approach.

- In summary, hyperglycemia aggravates ischemic AKI wherein Klotho is a potential connecting link. Klotho can be regulated by epigenetic (via HDAC inhibition) and non-epigenetic mechanisms (via ACE2 activation and ER stress inhibition) in ischemic AKI. Thus, by targeting these epigenetic and non-epigenetic mechanisms, Klotho loss and AKI progression can be prevented under type 1 diabetic and non-diabetic conditions. Hence, Klotho may prove as a promising therapeutic target against the ischemic AKI irrespective of diabetic conditions. However, a large scale and well-planned preclinical and clinical studies are required to further validate the therapeutic potential of these findings to translate this work for clinical use.

8. Limitations and future perspective

From the past two decades Klotho is in limelight of research being explored for its therapeutic potential against the various forms of kidney diseases. We have studied its mechanisms of regulations and evaluated its therapeutic potential against the AKI under diabetic and non-diabetic conditions. However, there were certain limitations which we were unable to address, which would have given more understanding of Klotho's therapeutic potential.

- Although we were successful to study the effects of different therapeutic interventions (TSA, DIZE, TUDCA) on endogenous Klotho expression and identifying its underlying molecular mechanisms, but we did not study the therapeutic benefits of exogenous Klotho in AKI. Since, Klotho has pleiotropic actions so the exogenous administration could have made significant value addition and impact on findings of this study.
- While, we have studied the effect of Klotho knockout *in-vitro*, we did not perform the same *in-vivo*. Effects of these pharmacological regimens in absence of Klotho *in-vivo* would have provided deeper information about its therapeutic importance against the AKI under diabetic and non-diabetic conditions.
- Thus, in future, the effects of exogenous Klotho alone and in combination with TSA, DIZE, or TUDCA will help to understand and further validate the reno-protective effects of Klotho in AKI under diabetic and nondiabetic conditions. Additionally, the effects of these therapeutic regimens in absence of Klotho (*in-vivo*) will further confirm the reno-protective effects of these regimens are Klotho dependent or not.

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Appendix I: Publications

List of publications from thesis

1. **Kale A**, Sankrityayan H, Gaikwad AB. (2022) Epigenetic restoration of endogenous Klotho expression alleviates acute kidney injury-diabetes comorbidity. *Life Sciences.* 288:120194 [IF: 6.1].
2. **Kale A**, Shelke V, Sankrityayan H, Dagar N, Gaikwad AB. (2022), Klotho restoration via ACE2 activation: A potential therapeutic strategy against acute kidney injury-diabetes comorbidity. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease.* 166532. [IF: 6.2].
3. **Kale A,** Sankrityayan H, Anders HJ, Gaikwad AB. (2021) Epigenetic and non-epigenetic regulation of Klotho in kidney disease. *Life Sciences.* 264:118644 [IF: 6.1].
4. **Kale A**, Sankrityayan H, Anders HJ, Gaikwad AB. (2021) Klotho: A possible mechanism of action of SGLT2 inhibitors preventing episodes of acute kidney injury and cardiorenal complications of diabetes. *Drug Discovery Today.* 1963-71 [IF: 7.4].
5. **Kale A,** Sankrityayan H, Anders HJ, Gaikwad AB. (2023) Klotho in kidney diseases: A crosstalk between the renin-angiotensin system and endoplasmic reticulum stress. *Nephrology Dialysis Transplantation.* 38(4):819-825 [IF: 6.2].
6. **Kale A,** Shelke V, Tahib H, Dagar N, Gaikwad AB. (2023) ER Stress Modulated Klotho Restoration: A Prophylactic Therapeutic Strategy Against Acute Kidney Injury-Diabetes Comorbidity" Submitted in the journal: *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease.* (Manuscript no: BBADIS-23-819 Status: under review).

List of national and international poster presentations from thesis:

1. **Kale A**, Shelke V, Habshi T, Dagar N, and Gaikwad AB, "Targeting ER Stress- Klotho Axis: A Novel Therapeutic Approach Against Ischemia-Reperfusion Renal Injury Under Type 1 Diabetes Conditions". At the International Symposium on Recent Trends and Future Opportunities in Pharmaceuticals, **PHARMACON-2022,** November 10-12, 2022, at NIPER Mohali, Punjab (INDIA).
2. **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". At the Conference on Recent Trends and Challenges in Drug Discovery", March 3-4, 2023, at BITS Pilani, Pilani Campus.

List of other publications

1. **Kale A,** Shelke V, Dagar N, Anders HJ, Gaikwad AB (2023) How to use COVID-19 antiviral drugs in patients with chronic kidney disease. *Frontiers in Pharmacology,* 14, 1053814. [IF: 5.6].
2. **Kale A,** Sharma A, Anders HJ, Gaikwad AB (2022) Diabetes and Cardiorenal Complications: A Clinical Review of Existing Therapies and Novel Combinations, Focusing on SGLT2 Inhibitors. *Current Diabetes Reviews.* 35975848.
3. **Kale A**, Anders HJ, Gaikwad AB. (2023) Lupus Nephritis: New and Emerging Biologic and Targeted Therapies. *BioDrugs*. 37, 463-475. [IF: 6.8].
4. Sankrityayan H, Shelke V, **Kale A,** Gaikwad AB. (2023) Evaluating the potential of tauroursodeoxycholic acid as add-on therapy in ameliorating streptozotocin-induced diabetic kidney disease. *European Journal of Pharmacology.* 20:175528. [IF: 5].
5. Sankrityayan H, **Kale A,** Shelke V, Gaikwad AB. (2022) Cyproheptadine, a SET7/9 inhibitor, reduces hyperglycemia-induced ER stress alleviating inflammation and fibrosis in renal tubular epithelial cells. *Archives of Physiology and Biochemistry*. 1:1-9. [IF: 3].
6. Sankrityayan H, **A Kale,** Gaikwad AB. (2021) 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*. 100 (3), 234-239. [IF: 2.245].
7. Tejeswini Sen T, **Kale A,** Lech M, Anders HJ, Gaikwad AB (2023), Promising novel therapeutic targets for kidney disease: Emphasis on kidney-specific proteins, *Drug Discovery Today*, 9;28(2):103466. [IF: 7.4].
8. Habshi T, Shelke V, **Kale A,** Anders HJ, Gaikwad AB (2023), Role of endoplasmic reticulum stress and autophagy in the transition from acute kidney injury to chronic kidney disease, *Journal of Cellular Physiology*, 238(1):82-93. [IF: 6.513].
9. Dagar N, **Kale A**, Steiger S, Anders HJ, Gaikwad AB (2022), Receptor-mediated mitophagy: An emerging therapeutic target in acute kidney injury, *Mitochondrion*, 66, 82-91. [IF: 4.534].
10. Shelke V, **Kale A**, Anders HJ, Gaikwad AB (2022) Epigenetic regulation of Toll-like receptors 2 and 4 in kidney disease. *Journal of Molecular Medicine* 7:1017-26. [IF 4.7].

11. Shelke V, **A Kale,** H Sankrityayan, HJ Anders, AB Gaikwad (2022) Long non-coding RNAs as emerging regulators of miRNAs and epigenetics in diabetes-related chronic kidney disease. *Archives of Physiology and Biochemistry*, 1-12 [IF 3].
12. Shelke V, **A Kale**, HJ Anders, AB Gaikwad (2022). Toll-like receptors 2 and 4 stress signaling and sodium-glucose cotransporter-2 in kidney disease. *Molecular and Cellular Biochemistry*, 36586092 [IF 4.3].
13. N Sharma, H Sankrityayan, **A Kale,** AB Gaikwad. (2020). Role of SET7/9 in the progression of ischemic renal injury in diabetic and non-diabetic rats. *Biochemical and Biophysical Research Communications.* 528 (1), 14-20 [IF 3.1].
14. Sankrityayan H, **Kale A,** Sharma N, Anders HJ, Gaikwad AB. (2020) Evidence for Use or Disuse of Renin-Angiotensin System Modulators in Patients Having COVID-19 With an Underlying Cardiorenal Disorder. *Journal of Cardiovascular Pharmacology and Therapeutics* 25(4):299-306. [IF: 2.6].
15. Habshi T, Shelke V, **Kale A,** Lech M, AB Gaikwad (2023), Hippo signaling in acute kidney injury to chronic kidney disease transition: current understandings and future targets. *Drug Discovery Today,* 103649. [IF 7.4].
16. Dagar N, **Kale A,** Jadhav HR, AB Gaikwad (2023), Nutraceuticals and network pharmacology approach for acute kidney injury: A review from the drug discovery aspect. *Fitoterapia*, 105563. [IF 3.4].
17. Shelke V, Yelgonde V, **Kale A**, Lech M, Gaikwad AB (2023), Epigenetic regulation of mitochondrial-endoplasmic reticulum dynamics in kidney diseases. *Journal of Cellular Physiology.* doi.org/10.1002/jcp.31058 [IF: 6.513].
18. Shelke V, **Kale A,** Dagar N, Gaikwad AB (2023), Concomitant inhibition of TLR-4 and SGLT2 by phloretin and empagliflozin prevents diabetes-associated ischemic acute kidney injury. *Food & Function.* 14 (11), 5391-5403. [IF: 6.1].

Appendix II: Biographies

Brief biography of the supervisor

Prof. Gaikwad Anil Bhanudas, is Professor and Head, Department of Pharmacy, Birla Institute of Technology and Science Pilani (BITS Pilani), Pilani Campus. 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. Till date, he has provided essential and novel evidences on histone post-translational modifications and the protective axis renin angiotensin system in the development of diabetic kidney diseases. He has contributed in several book chapters published by Elsevier and has 77 peer-reviewed research and review publications published in reputed international journals such as Drug Discovery Today (IF: 7.4), Cardiovascular Research (IF: 10.8), Pharmacological Research (IF: 9.3), British Journal of Pharmacology (IF: 7.3), BBA- molecular basis of disease (IF: 6.2). He has supervised one PDF and six Ph.D. students and, at present, is guiding one RA and four Ph.D. students. He is also serving an Associate Editor for Frontiers in Endocrinology Journal (Cardiovascular Endocrinology section) and BMC Pharmacology and Toxicology Journal. Further, he is also Review Editor for Renal Pharmacology section of the Frontiers in Pharmacology Journal.

Brief biography of the candidate

Mr. Kale Ajinath Vishwanath has completed his diploma in Pharmacy from SDMVM's institute of Pharmacy, Aurangabad, Maharashtra, India in 2014. He has pursued his bachelor of Pharmacy from Dr. Babasaheb Ambedkar Marathwada University (BAMU), Aurangabad, Maharashtra, India in the year 2017. He completed his M.S. Pharm. degree in Pharmacology and Toxicology from the National Institute of Pharmaceutical Education and Research (NIPER), Hyderabad, India in the year 2019. After masters he has worked as research trainee in Suven Life Sciences, Hyderabad from June-2019 to Janury-2020. Subsequently, he joined in the 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 acute kidney injury and other chronic kidney diseases in presence and absence of diabetes. During the thesis work he has mainly focused on Klotho regulation, epigenetics, renin-angiotensin system, endoplasmic reticulum stress in acute kidney injury under diabetic and non-diabetic conditions. Moreover, he has published 7 research and 17 review articles in various reputed, international, and peer-reviewed journals.