6. Discussion

6.1. Animal model standardization

Experimental surgery can be performed on the ventral (laparotomy) or the dorsal (retroperitoneal) regions of the body (Skrypnyk et al., 2013). To avoid the surgical stress occurred by ventral region surgery, dorsal region surgery is mostly preferred which has a faster recovery rate and higher chances of survival. During IRI, the ventral approach for renal surgery showed alteration in BUN levels due to delayed recovery which further reduced oral intake of fluids. However, sCr levels get less affected by hydration status (Skrypnyk et al., 2013). Clamping of renal arteries/vein or pedicle can be performed by following two major approaches- unilateral or bilateral. ***The bilateral clamping of renal arteries tends to influence the total renal mass and elevated the sCr and BUN levels within 24 h, which are the characteristic features of AKI in a clinical setup***. The bilateral IRI clamping is also utilized for studying renal fibrosis and CKD. It causes microvascular rarefication along with glomerular hypertrophy and interstitial scarring beyond ischemic insult (Holderied & Anders, 2014). However, the mouse model of AKI hinders the kidney regeneration analysis and associated long-term outcomes. On the other hand, shorter ischemia time points generally do not produce enough tubular necrosis to reach AKI criteria. As a choice, unilateral IRI is frequently used to study AKI to CKD transition by allowing the unilateral IRI animals for longer period reperfusion timings (Lech et al., 2009).

In this study, we utilized a unilateral model of renal ischemia to mimic the moderate form of AKI. Our study required a comparison between two different physiological conditions; DM and ND conditions. In DM rats, renal ischemia showed higher mortality compared to ND rats ***(Figure 5.1)***. Therefore, we used 45 min of unilateral renal ischemia followed by 48 h of reperfusion. In order to confirm AKI, we checked BUN levels and we found a significant increase in BUN levels in DM and ND rats. Unilateral IRI without contralateral nephrectomy have an intact kidney left after the surgical procedure, due to this, the possibility of mortality caused by kidney failure is greatly reduced, ultimately facilitates the assessment of AKI (Le Clef et al., 2016). Moreover, unilateral IRI also helps in conducting longer ischemic duration models (60 minutes in mice (Adachi et al., 2013) and

190 minutes in rats (Craddock, 1976)), hence permitting experiments in assessing a wide range of kidney injury stages (Le Clef et al., 2016). Therefore, the unilateral IRI model variant can induce histopathological kidney damage that closely related to the variability observed in patients (Salahudeen et al., 2004). Additionally, ischemic duration more than 60 minutes may result in acute tubular necrosis and renal failure, whereas ischemia of lesser than 30 minutes causes rapid proliferation of tubular epithelial cells and might revert the tubular injury (Williams et al., 1997).

On the other side, bilateral clamping of renal arteries is frequently been employed in the induction of ischemia in animals since it correlates to the pathophysiological conditions of human AKI, where the patient might have both the kidneys injured due to the impaired renal blood flow (Fu et al., 2018). The complications of AKI expressively augmented the mortality in critically ill ICU patients and dialysis has not appreciably lessened the death rate. While AKI is associated with high mortality, factors other than loss of kidney function appear to participate in poor outcomes (Grams & Rabb, 2012). These outcomes have been confirmed by comparing with dialysis-requiring patients with AKI which had considerably higher mortality than patients with end-stage renal disease. During AKI, an ischemic condition not only affect kidney function but also leads to distant organ dysfunction such as neurological, cardiac, and hepatic dysfunction. In various mice and rat IRI models, AKI results in the accumulation of systemic as well as tissue-specific inflammatory signaling molecules, activation of immune cells (monocytes, neutrophils) and ROS generation, ultimately precipitates distant organ dysfunction (Lee et al., 2018).

As per the clinical point of view, it is the most relevant model as a significant portion of in-hospital AKI remains ischemic in nature (Grams & Rabb, 2012). Therefore, in the third objective of this study, we utilized the bilateral IRI rat model to study AKI-associated distant organ dysfunction. Here, we performed 30 min or 20 min of bilateral IRI and the mortality rate was observed for up to 1 week. We found that 30 min of bilateral renal ischemia leads to higher mortality in comparison to 20 min of renal ischemia. Hence, we finalized 20 min bilateral IRI followed by 24 h of reperfusion. At 24 h of reperfusion time-point, we observed significantly elevated levels of BUN and PCr as compared to other time-points ***(Figure 5.2)***. Interestingly, these results are in parallel with previous reports (Choi et al., 2009; Kramer et al., 1999).

6.2. Role of SET7/9 in the progression of ischemic renal injury in diabetic and non-diabetic rats.

AKI is a frequent, progressive complication encountered in hospitalized patients for acute illness. Being having a vast spectrum, AKI ranging from minute alterations in the levels of biochemical markers of kidney function to kidney failure requiring dialysis. Compelling observations indicate that the prevalence of AKI is growing along with a bit decrease in mortality, however, associated complications might lead to long-term sequelae of AKI. The clinical evidence revealed the association of AKI with co-morbidities like diabetes. Despite different molecular and epigenetic mechanisms participated in the development of IRI are well demonstrated, only supportive therapies are popular against AKI, and the current remediation is scarce to prevent AKI. This arises a big question for a health concern, encourages a deeper understanding of the pathophysiology of AKI. ***It is a desideratum to focus on novel mechanisms that take part in the development of IRI. Therefore, we focused on histone methylation, H3K4-specific HMT i.e. SET7/9, involved the development of IRI under DM and ND rats.*** We found increased BUN levels which confirmed the renal functional impairment in IRI ***(Figure 5.3)***. In our study, we also observed the increased inflammatory NF-κB signaling and enhanced leukocyte infiltration ***(Figure 5.4)*** in ischemic ND and DM rats. Moreover, we also observed the augmented mRNA expressions of critical inflammatory mediators such as *Nf-κb, Mcp1* as well as *Tnfa* in ischemic ND and DM rats ***(Figure 5.4)***. These results are more pronounced in DM-IRI rats compared to ND-IRI rats. During IRI, cytokines and other pathological mediators remain strong intermediaries of NF-κB. Particularly, ischemic insult persuades the generation of TNF-α in an NF-κB-dependent manner, which in turns binds to TNF-α receptor to stimulate NF-κB activation. This induced a positive feedback mechanism for NF-κB regulation (Donnahoo et al., 1999; Zhang & Sun, 2015). Thus, this signaling cascade has a major contribution in the pathogenesis of IRI (Rabb et al., 2016; Zhang & Sun, 2015). Inflammation is also characterized by the recruitment of leukocytes, which is shown by the increased expressions of MCP-1 (Sung et al., 2002). Apart from it, the presence of hyperglycemia also triggers the inflammatory loop and progress the kidney damage (Song et al., 2019). Therefore, in our study, we can correlate that the increased protein and mRNA expressions of NF-κB under the presence of hyperglycemia, could be

involved in the renal ischemic damage by increasing cytokines and leukocyte infiltration ***(Figure 5.4)***.

As per our previous reports, Goru et al. have demonstrated the potential role of UPS in the development of type 1 diabetic nephropathy (Goru et al., 2016). Interestingly, this study also showed the crosstalk between histone methylation and histone ubiquitination in the progression of renal fibrosis. UPS is a vital degradation mechanism that controls the quality and function of numerous proteins, and any aberrations in UPS consequently result in the pathogenesis of diseases like neurodegenerative diseases, cancer, inflammatory diseases, and metabolic syndrome (Popovic et al., 2014). UPS not only ubiquitinates the substrate proteins that lead to 26 S proteasome-mediated degradation but it also involved in the regulation of numerous cell signaling pathways. The ubiquitination process is carried out by the consecutive action of activating (E1), conjugating (E2) and ligating (E3) enzymes, and it could be reversed by DUBs (Popovic et al., 2014). The dysregulation of UPS has been shown in diabetic renal disease. In our study, the increased expression of H2AK119Ub and H2BK120Ub in isolated proximal tubular cells of ischemic kidney ***(Figure 5.5)***, which might be related to the alterations in histone-specific UPS components like E3 ligases and DUBs. In proximal tubules, we found the increased mRNA levels of *Rnf2*- E3 ligase specific for H2AK120Ub decreased mRNA levels of DUBs like *Usp16, Usp21, Usp22.* Taken together, these results highlight the role of these UPS components (E3 ligases and DUBs) in the development of AKI.

Further, we checked the expression of H3K4Me2, H3K36Me2, and H3K9Me2 in ND-IRI and DM-IRI rats. As per growing evidence H3K4Me2, H3K36Me2 causes chromatin unwinding, thus facilitate the access of transcriptional machinery to the TATA box, whereas H3K9Me2 is correlated with gene silencing and transcriptional repression (Kouzarides, 2007). Our results showed the increased expression of H3K4Me2, H3K36Me2 while H3K9Me2 expression was significantly decreased in DM-IRI and ND-IRI rats ***(Figure 5.6)***. Unfortunately, due to the lack of tools, we were unable to prove the crosstalk of histone ubiquitination and histone methylation in AKI. And hence, we further focused on the vivacious role of histone methylation in AKI.

Furthermore, to check the inflammation and histone methylation crosstalk, we examined H3K4Me2, H3K9Me2, and H3K36Me2 in proximal tubules of DM-IRI and ND-IRI rats.

Increasing evidence shows that histone H3 methylation is involved in the pathogenesis of kidney diseases like diabetic kidney disease and ischemic-AKI. H3K4Me2 and H3K36Me2 are correlated with gene activation, while H3K9Me2 can be associated with gene silencing and transcriptional repression (Bannister & Kouzarides, 2011).In diabetic kidney diseases, H3K4Me2 showed increased enrichment at p65, TNF-α, and col1a1 gene (Brasacchio et al., 2009; Goru et al., 2016). H3K36Me2 showed higher levels at MCO-1 loci analyzed in the glomeruli from db/dbH2O mice compared with db/+H2O mice (Reddy et al., 2014). Isolated glomeruli from diabetic nephropathy rats depicted the higher levels of H3K9Me2 at the col1a1 gene (Goru et al., 2016). In addition, Vascular smooth muscle cells (VSMCs) derived from diabetic db/db mice, showed decreased occupancy of H3K9Me2 at inflammatory gene promoters (Villeneuve et al., 2008). These facts advocate that the enhancement of H3K4Me and suppressed repressive H3K9Me mark can upregulate the expression of pathological genes under diabetic kidney disorders. In our study, H3K4Me2 and H3K36Me2 expression were found to be increased in isolated proximal tubules, where H3K9Me2 expression was reduced in proximal tubules of DM and ND rats ***(Figure 5.6)***. However, the abovementioned results were highly prominent in DM-IRI rats ***(Figure 5.6)***. Next, we checked the protein expression of HMT-SET7/9 in proximal tubules of ND-IRI and DM-IRI rats. SET7/9 is mostly involved in H3K4Me2 (Ruthenburg et al., 2007). In a cell culture study, silencing of SET7/9 gene with small interfering RNAs in monocytes markedly inhibited TNF-α induced inflammatory genes and H3K4Me2 on the TNF-α promoters (Li et al., 2008b). Sasaki et al. showed that knockdown of SET7/9 expression with small interfering RNA significantly attenuated renal fibrosis in the UUO mice (Sasaki et al., 2016). In addition, renal mesangial cells showed enhanced H3K4me1/3 expression and SET7/9 occupancies at the p21 promoter under diabetic condition (Li et al., 2016b). These studies highlighted that SET7/9 are potential therapeutic targets in preventing IRI under DM as well as ND rats. In our study, we found that renal ischemic insult caused increased protein and mRNA expressions of SET7/9 in DM and ND rats. However, these results are more significant in DM-IRI rats compared to ND rats. In recent studies, researchers have demonstrated the connection of increased expression of SET7/9 expressions and increased inflammatory cascade in diabetes and AKI conditions. It provides an insight towards the use of SET7/9 inhibitor under these conditions. Sinefungin,

a SET7/9 inhibitor, ameliorated the renal fibrosis by inhibiting TGF-b1 and H3K4me1 in both cell lines (NRK-52E and NRK-49F cells) and UUO mice (Sasaki et al., 2016). Recently, Cyproheptadine, a novel SET7/9 inhibitor has been reported to exert protective role against breast cancer (Takemoto et al., 2016). SET7/9 has claimed to methylated non-histone proteins including estrogen receptor (ER)-α. ER-α methylation activates the pathogenic transcriptional activities and precipitates the carcinogenesis of breast cancer. Cyproheptadine, clinically approved antiallergy drug, used as a SET7/9 inhibitor, which hinders the substrate-binding pocket of Set7/9 along with its enzymatic activity via competing with the methyl group acceptor. Hang et al. demonstrated the Cyproheptadine reduced cancer-induced bone pain via decreasing spinal SET7/9 and RANTES expression. Administration of SET7/9 (0.2 μg) in mice significantly abolished the anti-nociceptive effects of Cyproheptadine, proved the selectively of the same for SET7/9 (Hang et al., 2017). In our study, we used Cyproheptadine against IRI in DM and ND rats. We found that high dose of Cyproheptadine has effectively improved the renal functions via reducing BUN levels and tubular necrosis in DM-IRI and ND-IRI rats ***(Figure 5.7; Figure 5.8)***.

NF-κB pathway and its regulated inflammatory genes, such as TNF-α and MCP-1, join up with the pathogenesis of inflammatory diseases, involving diabetes and AKI (Markó et al., 2016; Shanmugam et al., 2003). Growing evidence reported the essential role of HMT-SET7/9 in regulating NF-κB-dependent genes and chromatin H3-K4Me induced TNF-α and S100b, a ligand of the RAGE receptor for advanced glycation end products. Whereas, down-regulation of SET7/9 in monocytes reported to decrease the expression of key NF-κB-dependent genes induced by inflammatory stimuli, demonstrated a novel biological role for SET7/9 and H3-K4Me in inflammation and diabetes (Li et al., 2008b). Additionally, SET7/9 also regulates renal fibrosis in type 1 diabetic condition (Goru et al., 2016). In the streamline of the above-mentioned studies, we observed a significant increase in the expressions of p-NF-κB ***(Figure 5.9)*** and MCP-1 ***(Figure 5.10)*** in DM and ND ischemic kidneys. These alterations were found to be more prominent in DM-IRI kidney compared to ND-IRI rats. Moreover, the Cyproheptadine treatment has ameliorated the p-NF-κB and MCP-1 expressions in ischemic kidneys ***(Figure 5.9; Figure 5.10)***. As compared to low dose treatment, a high dose of Cyproheptadine has a significant effect in attenuating the expressions of inflammatory markers (p-NF-κB and MCP-1). Additionally,

Cyproheptadine treatment has effectively reduced SET7/9 expression in the ischemic kidney as compared to DM-IRI and ND-IRI kidneys ***(Figure 5.11)***. Taken together, these results showed SET7/9 as a promising target which regulates NF-κB-mediated inflammation via upregulating methylation at H3K4 under IRI in DM and ND conditions ***(Figure 6.1)***. for the first time, we demonstrated that Cyproheptadine has effectively prevented the IRI in DM and ND condition, and provides a vast idea to conduct pathological studies exploring HMT-SET7/9 which is a novel and enticing target under the same. In addition, Cyproheptadine is an clinically approved anti-allergic drug, therefore, it could be repurposed for the AKI treatment.

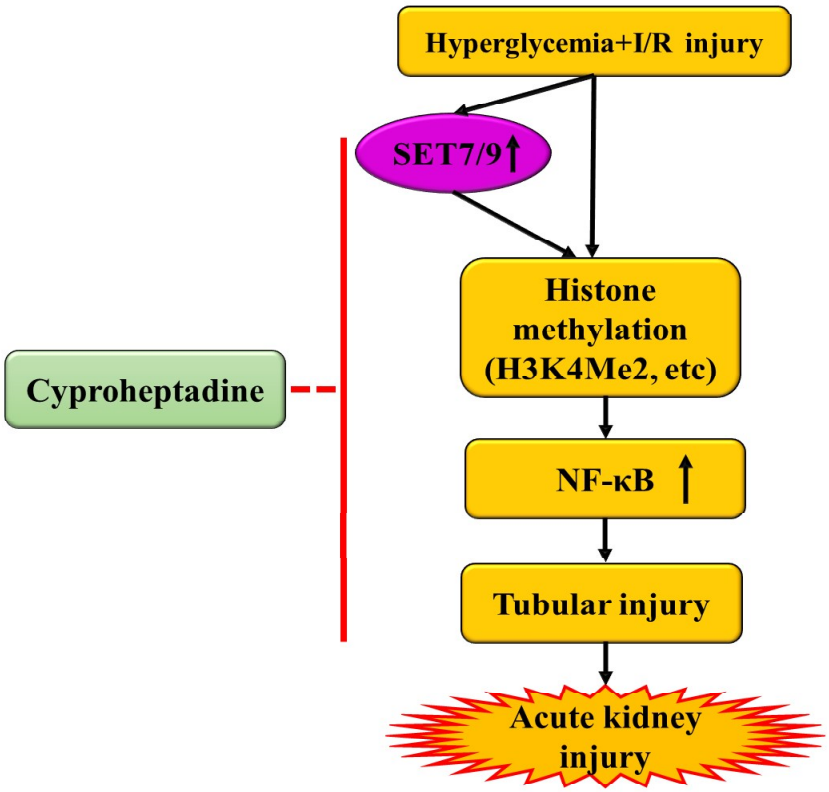

Figure 6.1 Possible mechanisms behind the reno-protective role of Cyproheptadine on AKI under diabetic and non-diabetic conditions.

In diabetic and non-diabetic kidneys, increased expression of SET7/9, H3K4Me2, NF-κB, and severe tubular damage was observed and all these alterations were reversed by Cyproheptadine administration. This is the novel mechanism of Cyproheptadine, in preventing ischemic renal injury in diabetic and non-diabetic animals. I/R- Ischemia-reperfusion.

6.3. Angiotensin-II type 2 receptor and angiotensin-converting enzyme 2 mediate ischemic renal injury.

We hypothesized that the depressor arm of RAS plays a major role in IRI under both diabetic and non-diabetic conditions. Our result demonstrated augmented AT1R, ACE, and Ang II expressions, as well as AT2R and reduced ACE2 and Ang (1-7) expression in proximal renal tubules of DM and ND rats subjected to IRI. Interestingly, the administration of AT2R agonist (C21) and ACE2 activator (Dize) per se marginally ameliorated pathological changes associated with IRI including metabolic perturbation, increased renal tubular cells oxidative stress, apoptosis, and inflammation. However, their combination therapy significantly normalized the alterations in RAS components, thereby attenuated the above-mentioned pathological consequences associated with IRI in diabetic and non-diabetic rats.

The epidemiologic reports advocated that AKI is more lethal in diabetic patients in comparison to non-diabetic individuals; however, responsible molecular mechanisms are still elusive (Thakar et al., 2011). In the present study, two weeks after STZ-injection, DM rats were subjected to I/R to induce AKI, we used this experimental animal model to mimic the pathophysiology of comorbid diabetes and AKI in humans. We observed that IRI increased BUN levels and oxidative stress in DM rats compared to ND rats ***(Table 5.1, Figure 5.12)***. Existing literature has speculated that critical pathogenic factors of AKI comprise compromised kidney perfusion and altered the intrarenal hemodynamic balance, which largely attributed to systemic and intrarenal RAS activation (Matejovic et al., 2016). Individually, hyperglycemia or IRI increased levels of Ang II; an octapeptide, and the major effector of RAS pressor arm mediating pathological effects via activation of AT1R (Malek & Gaikwad, 2017). ***Consistent with this, we observed activation of the pressor arm of the RAS demonstrated by increased Ang II, ACE and AT1R expression in a renal proximal tubular fraction from kidneys of diabetic rats that underwent IRI*** ***(Figure 5.13, 5.14)***.

On the other hand, the depressor arm components: AT2R, ACE2, Ang (1-7) are identified for their positive feedback mechanism by recognizing elevated cellular stress and activated pathological signaling (da Silveira et al., 2010) (Goru et al., 2017b). Male Wistar rats subjected to left nephrectomy and 45 min ischemia on right kidney demonstrated reduced

renal ACE2 mRNA expression and Ang (1-7) levels at 4h reperfusion (da Silveira et al., 2010). In subtotal nephrectomized rats, Dize (ACE2 activator) augmented cortical and medullary ACE2 activity and abridged cortical ACE activity (Velkoska et al., 2015). Recently, we have reported that Dize monotherapy (5 mg/kg/day) marginally attenuated diabetic renal fibrosis, whereas Dize in combination with a neprilysin inhibitor thiorphan significantly attenuated the development of diabetic nephropathy (Malek et al., 2019b). Further, C21 has demonstrated to halt the development of diabetic nephropathy in mice and rats (Koulis et al., 2015). Recently, we reported that C21 monotherapy at dose 0.3 mg/kg/day has partially improved renal functions, while C21 with Telmisartan combination has markedly mitigated diabetic nephropathy by attenuating apoptotic signaling (Goru et al., 2017b).

In the present study, we observed reduced tubular ACE2 and Ang-(1-7) expressions and a compensatory increase in AT2R expression in DM-IRI rats when compared to DM and ND-IRI rats. Hence, we speculated that modulating the RAS depressor arm by ACE2 activator or AT2R agonist might protect the kidney against IRI. On the basis of the abovementioned reports, we treated ND-IRI and DM-IRI rats with AT2R agonist, C21 (0.3 mg/kg/day, *i.p.*) or ACE2 activator, Dize (5 mg/kg/day, *p.o.*) for five days. Our data suggest that the monotherapy using either AT2R agonist or ACE2 activator in ND-IRI and DM-IRI rats had an only minor effect on tubular damage as evident from oxidative stress parameters and histopathological evaluations. Hence, we plan to administer AT2R agonist and ACE2 activator together as a combination therapy at the same dose regimen in ND-IRI and DM-IRI rats. We found that concomitant AT2R agonism and ACE2 activation significantly attenuated oxidative stress associated with IRI in ND and DM rats ***(Figure 5.15)***. Further, IRI has been reported to activate antioxidant transcription factor Nrf2 (Leonard et al., 2006). In this regard, we observed increased Nrf2 expression in a renal tubular fraction of ND-IRI and DM-IRI rats. Interestingly, C21 and Dize combination therapy to ND-IRI and DM-IRI rats further amplified proximal tubular Nrf2 expression ***(Figure 5.15)***. Interestingly, simultaneous AT2R agonist and ACE2 activator administration have attenuated proinflammatory cytokines *Il6, Tnfa,* and *Mcp1* mRNA expressions and preventing NF-κB signaling-mediated inflammation, MCP-1-mediated leukocyte infiltration, and c-Cas-3 and cPARP-mediated apoptosis in the proximal tubular

fraction of ND-IRI and DM-IRI rats ***(Figure 5.15, 5.16)***. Previous studies showed that C21 in combination with telmisartan or losartan alleviated glomerular damage, extracellular matrix accumulation, and increased glomerular nephrin expression in type 2 diabetic rats (Goru et al., 2017b). Similarly, we observed that C21, AT2R agonist along with Dize, ACE2 activator was better in mitigating morphological alterations (tubular necrosis) when compared to monotherapies ***(Figure 5.17)***. Moreover, ELISA results revealed the superiority of C21 and Dize combination therapy over respective monotherapies in normalizing systemic (plasma) and local (proximal tubules) alteration in RAS components level associated with IRI in ND and DM rats ***(Figure 5.18)***.

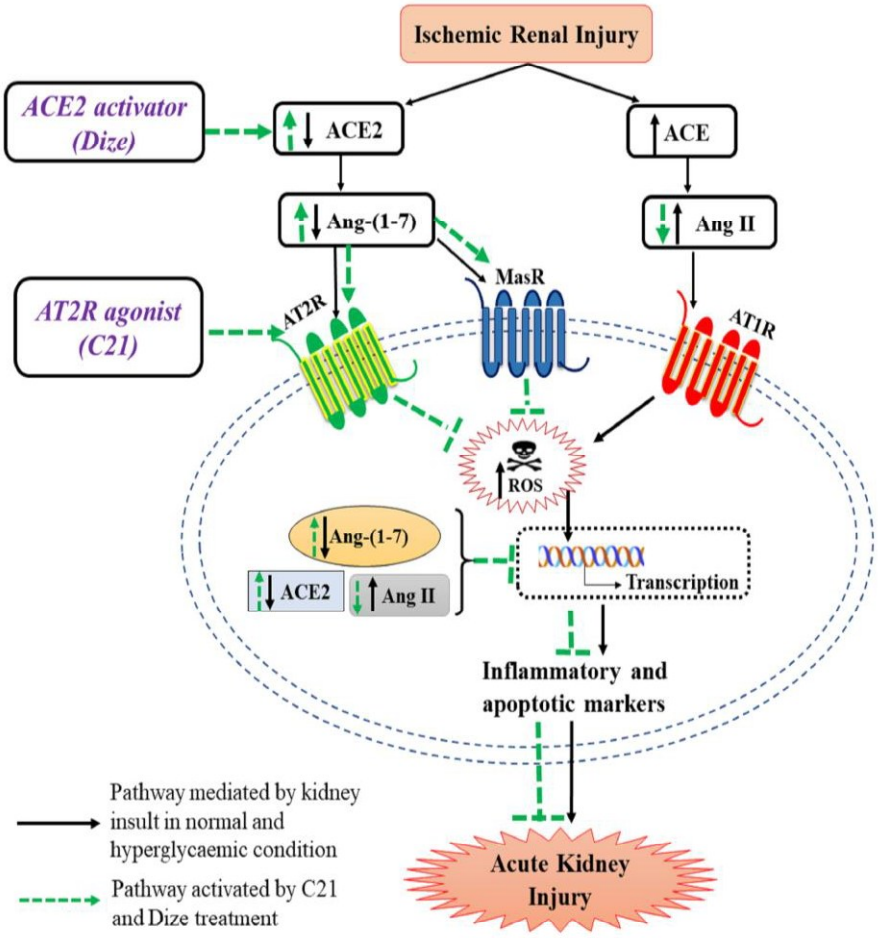

Figure 6.2 AT2R and ACE2 activation attenuated acute kidney injury.

C21 and Dize combination therapy prevented ischemic renal injury induced renal abnormalities such as decreased ACE2 activity, ACE2 expression in proximal tubules, Ang (1-7) levels and increased AT1 receptors, oxidative stress, inflammatory and apoptotic markers by acting through ACE2/Ang (1-7)/ AT2 axis.

The combination therapy further augmented mRNA expressions of renoprotective AT2R and MasR in IRI subjected ND and DM rats. In brief, these results provide us with articulate evidence that AT2R and ACE2 are involved in the pathogenesis of ischemic AKI. In this study, elevated inflammatory, apoptotic, and another pathogenic signaling can be correlated with dysregulated RAS depressor arm (reduced Ang (1-7) and ACE2 levels) and severity of ischemic AKI. ***For the first time, we stated that the novel combination of AT2R agonist and ACE2 activator has significantly attenuated the IRI related kidney impairments in DM and ND rats (Figure 6.2).***

6.4. Activation of angiotensin II type 2 receptor and angiotensin-converting enzyme 2 attenuates ischemic renal injury-associated distant organ dysfunction

6.4.1. AT2R agonist and ACE2 activator diminished IRI-induced brain dysfunction

Alteration in RAS plays a major role in neurological sequelae under diabetes and various kidney diseases (Cao et al., 2015b; Padda et al., 2015). Individually, diabetes and AKI episodes reported causing neurological dysfunction via disruption of the blood-brain barrier, activation of the inflammatory cascade, derangement of neurotransmitters, and altercation in RAS arms (Cao et al., 2017; Liu et al., 2008; Lu et al., 2015). Cao et al. have established the role of the reno-cerebral RAS pathway under AKI as well as CKD (Cao et al., 2017; Cao et al., 2015b).

Recently, the depressor arm of RAS has gained popularity as an important molecular target involved in various neurological dysfunctions like ischemic stroke and cognitive impairment (Ahmed et al., 2019; Bennion et al., 2017). ***However, the role of depressor arm in the pathophysiology of AKI abides poorly understood. Therefore, in the present study, we investigated the role of the depressor arm of RAS on the neurological dysfunction induced by AKI in non-diabetic and diabetic rats.*** We found that IRI simultaneously activated the reno-cerebral RAS in ND and DM rats. The damaged kidneys and brain showed marked elevated inflammation markers level that impaired the cognitive behavior of ND and DM rats ***(Figure 5.19)***. The alteration in reno-cerebral RAS leads to activation of the pressor arm that results in increased Ang II levels and suppression of depressor arm causes depletion of Ang (1-7) levels in renal and hippocampal tissue.

Interestingly, the above-mentioned outcomes were significantly pronounced in DM-IRI rats compared to ND-IRI rats.

Treatment with the combination of two major depressor arm modulators, C21 (0.3 mg/kg/day, *i.p.*) and Dize (5 mg/kg/day, *p.o.*), attenuated the IRI induced increased in BUN, PCr and urinary KIM-1 levels in ND and DM rats. Thus, suggesting the potential of AT2R agonist and ACE2 activator in normalizing the kidney functional parameters ***(Figure 5.20)***. Similar results were obtained in previous reports, where improved BUN and PCr levels were observed in IRI and diabetic rats as a result of Dize administration (Goru et al., 2017b). Both AKI and diabetes have been reportedly elevated the cerebral oxidative damage (Etienne et al., 2019; Kovalčíková et al., 2018). Increased brain oxidative stress markers (MDA, nitrite levels) most prominently found in the hippocampal region of the brain after IRI episode (Kovalčíková et al., 2018). In this study, DM-IRI rats showed a marked increase in MDA and nitrite levels in isolated proximal tubular and hippocampal homogenates compared to ND-IRI rats. Combination therapy of C21 and Dize has significantly attenuated the increased MDA and nitrite levels in ND-IRI rats. However, a similar suppression was not observed in DM-IRI rats ***(Figure 5.21)***. Next, we performed behavioral assessment using an actophotometer in DM-IRI and ND-IRI rats. IRI has severely impaired locomotor activity in DM rats compared to ND rats. Regarding the mechanism of altered motor activity, increased uremia leads to change in monoamine metabolism (specifically dopamine) and further altered motor activity (Adachi et al., 2001; Verma et al., 2018). The connecting link of the reno-cerebral RAS axis remains another possibility to alter motor activity in IRI rats (Cao et al., 2017). Combination therapy of C21 and Dize has improved the motor activity to a greater extent in ND rats compared to DM rats ***(Figure 5.21)***.

Histopathological evaluation by H and E staining revealed elevated pyknotic neuronal cells in CA1 of the hippocampus. The hippocampus imparts a kin role in learning and memory as well as in anxiety and depression (Anacker & Hen, 2017; Hollands et al., 2016). Selectively, the CA1 region remains the highly vulnerable hippocampal part to degenerate in various pathological anomalies (Bordi et al., 2016; Lee et al., 2017a). Previous reports in Alzheimer's disease and diabetes showed the existence of CA1 pathology and inflammation along with hypoactivity in actophotometer (Sharma et al., 2015; Wei et al.,

2019). Therefore, IRI induced damage to hippocampal CA1 neurons could account for the hypoactivity of the rats. Whereas, concomitant therapy of C21 and Dize showed significant alleviation of neuronal damage in these regions in ND rats compared to DM rats ***(Figure 5.22)***.

GCSF is a hematopoietic growth factor that regulates the proliferation and differentiation of neural stem cells. Studies have revealed its neuroprotective role under Alzheimer's disease and cerebral ischemia (Li et al., 2015; Wu et al., 2017). In our study, IRI results in elevated circulating and hippocampal GCSF levels in ND and DM rats. However, none of the treatments has further enhanced the GCSF levels. In higher vertebrates, brain injury resulted from chemical insult, trauma, genetic disorders or AKI, consequently elevated the reactive astrocytes which increased the GFAP synthesis in the cerebral cortex, hippocampus, GFAP considered as inflammatory sign in brain tissue and is proved by protein levels and immunostaining GFAP (Cao et al., 2017; Liu et al., 2008). Similarly, we also observed the elevated GFAP protein levels in both plasma and cerebral cortices of DM-IRI rats compared to ND-IRI rats. After combination therapy, ND rats showed a marked reduction in GFAP levels compared to DM rats ***(Figure 5.23)***.

Both clinical and laboratory reports evidenced that IRI results in an upsurge of brain inflammation via over-expressing of pro-inflammatory cytokines such as keratinocyte derived chemoattractant (KC), IL-1, TNF-α, macrophage inflammatory protein (MIP)-1, and MCP-1 (Lee et al., 2018). With respect to these results, our study also showed increased TNF-α expression evaluated by immunostaining. In ND rats, combination therapy significantly attenuated the TNF- α levels as compared to DM rats ***(Figure 5.24)***. These results are in streak with previous studies indicating the ability of AT2R and ACE2 stimulation to reduce inflammatory cytokines production and mediate neuroprotective role in cerebral stroke and Alzheimer's Disease (Kamel et al., 2018; Min et al., 2014).

Available literature established a mechanistic link between the down-regulation of RAS depressor arm [AT2R/ACE2/Ang (1-7)/MasR axis] and neurological dysfunction (Bennion et al., 2017; Kangussu et al., 2017). In this regards, depressor arm modulators strongly proved their neuroprotective role in vascular stroke, Alzheimer's disease, and anxiety conditions by virtue of increasing AT2R, ACE2, Ang (1-7) and MasR expressions (Chen et al., 2014b; Dai et al., 2015; Füchtemeier et al., 2015; Wang et al., 2016). In line with

these findings, we first examined the RAS components in the renal proximal tubules and urine samples. In proximal tubules, IRI resulted in a significant elevation of ACE, Ang II levels, and marked reduction of Ang (1-7) and ACE2 levels of DM rats compared to ND rats. However, combination treatment significantly reduced the ACE, Ang II levels, and augmented the Ang (1-7), ACE2 levels in ND and DM rats. On the other hand, increased urinary AGT levels were markedly reduced with concomitant AT2R and ACE2 stimulation in ND and DM rats ***(Figure 5.25)***. IRI reported to stimulate a sympathetic reflex that interconnects the renal and cerebral RAS and promotes the free radical generation, eventually progression of renal injury (Cao et al., 2017). Similarly, we observed the effect of IRI on hippocampal RAS and found an elevation in pressor arm and suppression of depressor arm components in ND and DM rats. Combination therapy has effectively normalized the Ang II levels, however; Ang (1-7) protein levels, At2r and MasR mRNA expressions were significantly upregulated in ND and DM rats. Interestingly, we found that the aforesaid results were more distinct in ND rats compared to DM rats ***(Figure 5.26)***.

The current study is aimed to explore the neuroprotective benefits of targeting the depressor arm of RAS against the acute responses of IRI. Recent epidemiological and experimental data have provided substantial information that AKI imparts to the development and progression of chronic kidney diseases and its associated distant organ dysfunction such as neurological dysfunction (Basile et al., 2016; Chawla et al., 2014; Ferenbach & Bonventre, 2015; Shiao et al., 2015; Venkatachalam et al., 2015). ***Though, it remains a notable query of the impact of depressor arm modulators (C21 and Dize) treatment on longer-term neurological outcomes, which is the limitation of the present study. Therefore, we expect that future studies are required in this area where substantial changes would be detected at later time points post-IRI and will determine the effects of C21 and Dize under such pathological conditions.***

It has been proven difficult to target CNS with pharmacotherapy because the BBB prohibits several molecules and restricts their activity centrally (Pardridge, 2005). Altogether, small and lipophilic molecules having molecular mass less than 400-500 g/mol crosses BBB (Ghose et al., 1999). However, BBB disruption occurs following ischemic renal insult, which consequently increases BBB permeability (Nongnuch et al., 2014). Regarding the drug distribution profile, C21 and Dize effectively circulate among the kidney, heart, liver,

and brain (Kuriakose & Uzonna, 2014). Interestingly, intraperitoneal administration of C21 and Dize has already been reported for their neuroprotective role either directly, or by the anti-inflammatory action (secondary neuroprotection) understroke and Alzheimer's disease (Bennion et al., 2015; Evans et al., 2020). As the current study is limited to the peripheral treatment strategy for IRI-associated neurological dysfunction, future studies focusing on the intracerebroventricular administration of C21 and Dize would provide crucial neurological data which would be more direct to the brain. ***In summary, to the best of our knowledge, this is the first report, demonstrating the reno-protective effect of AT2R agonist-C21 in combination with ACE2 activator-Dize against the IRI-induced brain dysfunction under hyperglycemic condition (Figure 6.3).***

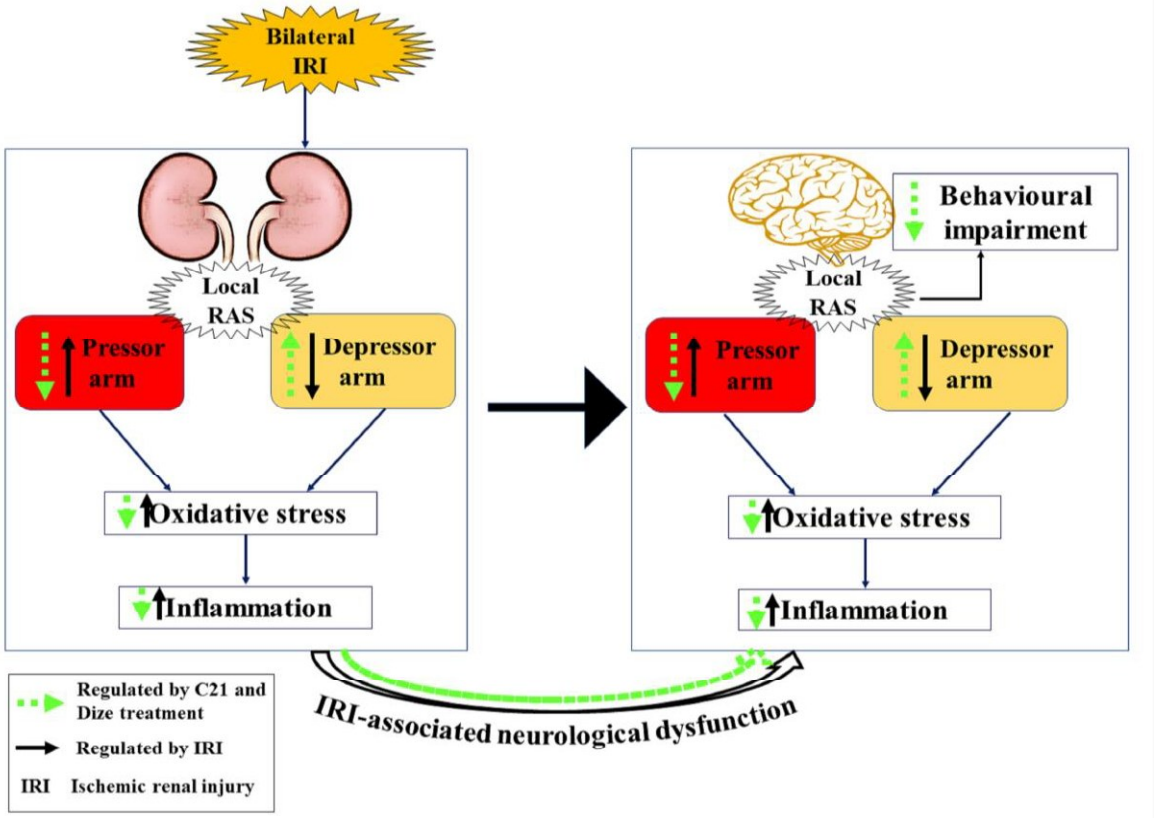

Figure 6.3 AT2R and ACE2 activation prevented IRI-induced brain dysfunction

The beneficial role of AT2R agonist-C21 and ACE2 activator-Dize in IRI-associated neurological dysfunction exerts through preventing oxidative stress, inflammatory markers, and upregulating brain depressor arm axis of RAS under diabetic and non-diabetic conditions.

6.4.2. Amelioration of ischemic renal injury associated cardiac and hepatic dysfunction: Role of AT2R and ACE2

Clinical reports have cleared that mortality rate soars from 45% to 60% when AKI associated with distant organ insufficiency and co-morbidities (such as diabetes) (Lee et al., 2018). As compared to non-diabetics, diabetic patients endure a higher risk of AKI under critically ill hospitalized conditions (Hursh et al., 2017). ***Additionally, heart and liver dysfunction in IRI inadequately tacit clinical condition, due to the complexity and insufficient understanding of pathological signals*** (Husain-Syed et al., 2019). Nevertheless, studies have revealed the possible mechanisms behind the multiorgan dysfunction under IRI settings, including vascular inflammation, metabolic acidosis, electrolyte imbalances triggered life-threatening arrhythmias, reactive oxygen species (ROS), upregulation of inflammatory cytokines and pro-apoptotic molecules (Bucsics & Krones, 2017; Lee et al., 2018). Besides these distinctive mechanisms, there is an imperative factor with immediate impact on distant organs, i.e., renin-angiotensin system (RAS) (Bucsics & Krones, 2017; Panico et al., 2019).

Recently, the depressor arm of RAS has gained acceptance as an essential molecular target involved in various cardiac and hepatic dysfunctions like diabetic cardiomyopathy, pulmonary hypertension, and NAFLD (Cao et al., 2019; Macedo et al., 2016). Although, its role in the pathophysiology of IRI-associated cardiac and hepatic dysfunctions is still elusive. ***Therefore, in the present study, we aimed to investigate the role of the depressor arm of RAS in IRI-associated cardio-hepatic dysfunction under normal and hyperglycemic conditions.*** Given due weightage to existing literature and our previous reports, we have designed the treatment groups with depressor arm modulators, C21 (0.3 mg/kg/day, *i.p.*) and Dize (5 mg/kg/day, *p.o.*) monotherapies as well as their combination therapy to evaluate their efficacy against IRI in ND and DM male Wistar rats. The IRI rats of ND and DM groups exhibited substantial renal functional decline, as shown by plasma and urine perturbations, such as BUN, PCr, and urinary KIM-1 levels which were significantly attenuated by proposed combination therapy. These results suggested the potential of AT2R agonist and ACE2 activator in improving kidney functional parameters ***(Table 5.3)***. A similar pattern of the result was obtained in previous reports, where BUN

and PCr levels were significantly improved in the IRI rat and diabetic model as a result of C21 and Dize administration (Goru et al., 2017b; Pandey & Gaikwad, 2017b).

According to previous reports, IRI cause the release of LDH and CK-MB into the bloodstream from damaged tissues signified as myocardial injury markers (Amini et al., 2019; Mohammadi et al., 2019). In concurrence with existing reports, the present study demonstrated that the IRI induced AKI to cause myocardial injury which manifested itself as raised systemic activities of LDH, CK-MB as compared to control groups ***(Table 5.3)***. However, these biochemical indicators got significantly normalized by AT2R and ACE2 agonist combination therapy. Moreover, the SBP level was not altered by IRI which was in agreement with previous reports (Kelly, 2003) ***(Table 5.3)***.

Scientists have claimed the elevation of liver injury biochemical indicators as a result of severe IRI under clinical as well as preclinical settings (Lai et al., 2019; Sun et al., 2012). Similarly, in our report, liver injury markers such as activities of AST and ALT were significantly elevated after IRI in ND and DM rats which further markedly normalized with the combination treatment of C21 and Dize ***(Table 5.3)***.

The subsequent mechanism for the progression of cardio-hepatic dysfunction involves a combination of oxidative stress, lipid peroxidation mechanisms under AKI, and diabetes conditions (Abdellatief et al., 2017; Bigagli & Lodovici, 2019). In experimental studies, the initiation and perpetuation of cellular injury in IRI affected cardiac and hepatic tissue, is associated with the upsurge in free radicals and the depletion of endogenous antioxidant defense (Amini et al., 2019) (Fadillioglu et al., 2008). MDA, an index of lipid peroxidation, whereas GSH and catalase, indexes of anti-oxidant defense mechanism, have been shown to increase in remote cardiac and liver injury augmented by IRI (Park et al., 2012; Yap & Lee, 2012; Zhao et al., 2018). In the current study, we found that simultaneous AT2R and ACE2 activation able to control the abrupt rise in free radical stress by restricting lipid peroxidation and normalizing GSH and catalase activity in the heart and liver tissue of ND/DM-IRI rats ***(Figure 5.27)***. These findings may be attributed to the diminution of lipid peroxidation and supported by previous reports, which demonstrated the potential anti-oxidant activities of C21 and Dize (Hrenak et al., 2013; Omoniwa et al., 2019).

MPO, a neutrophil-specific enzyme referred to as an indicator of leukocyte infiltration and utilized to define the role of neutrophils in various types of tissue injuries, including remote

organ injuries (Brochner et al., 2014; Kelly, 2003). In our study, the elevated aforementioned cardiac and hepatic oxidative stress is accompanied by increased MPO activity in IRI groups of ND and DM rats ***(Figure 5.27)***. This increased MPO activity was significantly controlled by the administration of C21 and Dize combination therapy. These findings are in the streamline with the studies explaining the AT2R and ACE2 activation, provides diminished cardiac and hepatic MPO activity (Kaschina et al., 2008; Souza et al., 2016). In the heart, H and E staining of IRI groups showed severe myocardial degenerative changes, which were identified by disarrayed cardiac muscle fibers ***(Figure 5.28)***. However, liver histology of IRI groups showed sinusoidal dilatation and vacuolization of hepatocytes along with leukocytes infiltration, pyknotic nuclei, and Kupffer cell proliferation ***(Figure 5.29)***. Previous reports of AKI showed the existence of cardio-hepatic pathology and inflammation (Abdellatief et al., 2017). In contrast, concomitant therapy of C21 and Dize revealed significant alleviation of cardio-hepatic structural damage in IRI groups of ND and DM rats.

Overexpression of TNF-α was observed within the hepatocytes and cardiomyocytes in IRI rats. These alterations are in agreement with those reported by Panico et al. (Panico et al., 2019), Serteser et al. (Serteser et al., 2002) and Lai et al. (Lai et al., 2019). Overexpression of TNF-α in IRI injured tissue may be related to IRI-induced inflammation (Lai et al., 2019). In our lab, we have previously studied the potential anti-inflammatory effect of C21 and Dize in diabetes-related microvascular complications (Malek et al., 2019a). Moreover, we have also demonstrated that treatment with C21 and Dize at the time of renal ischemia results in the preservation of renal function. Hence, an alternate explanation for the lack of an increase in IRI-induced cardiac and hepatic TNF-α expression after C21 and Dize administration is the lack of kidney failure in this setting ***(Figure 5.30, 5.31)***.

Numerous studies have provided mechanistic evidence that the depressor arm of RAS is required for suitable cardiac and hepatic function. These studies proposed that the repression of the AT2R/ACE2/Ang (1-7)/MasR axis showed hindered functions of the same (Santos et al., 2006; Silva et al., 2013). Nevertheless, depressor arm modulators strongly reveal their cardio-hepatic protective role in hypertension, atherosclerosis, insulin resistance (Chow et al., 2016; Quiroga et al., 2018; Rajapaksha et al., 2018) as evidenced by increased ACE2, Ang (1-7), AT2R and MasR expressions. Following these findings,

we first examined the RAS components in the systemic circulation and in the urine. IRI results in a marked reduction of ACE2 and Ang (1-7) levels followed by a significant increase in ACE, Ang II, and urinary AGT levels in DM rats as compared to ND rats. However, combination therapy normalized the pressor and depressor arm components in plasma as well as urine ***(Figure 5.32, 5.33)***. A most recent study revealed the novel mechanism of kidney-heart interaction under the IRI condition and suggested that cardiac inflammatory cascades triggered by the simultaneous upregulation of sympathetic reflex and RAS in the cardiac tissue (Panico et al., 2019).

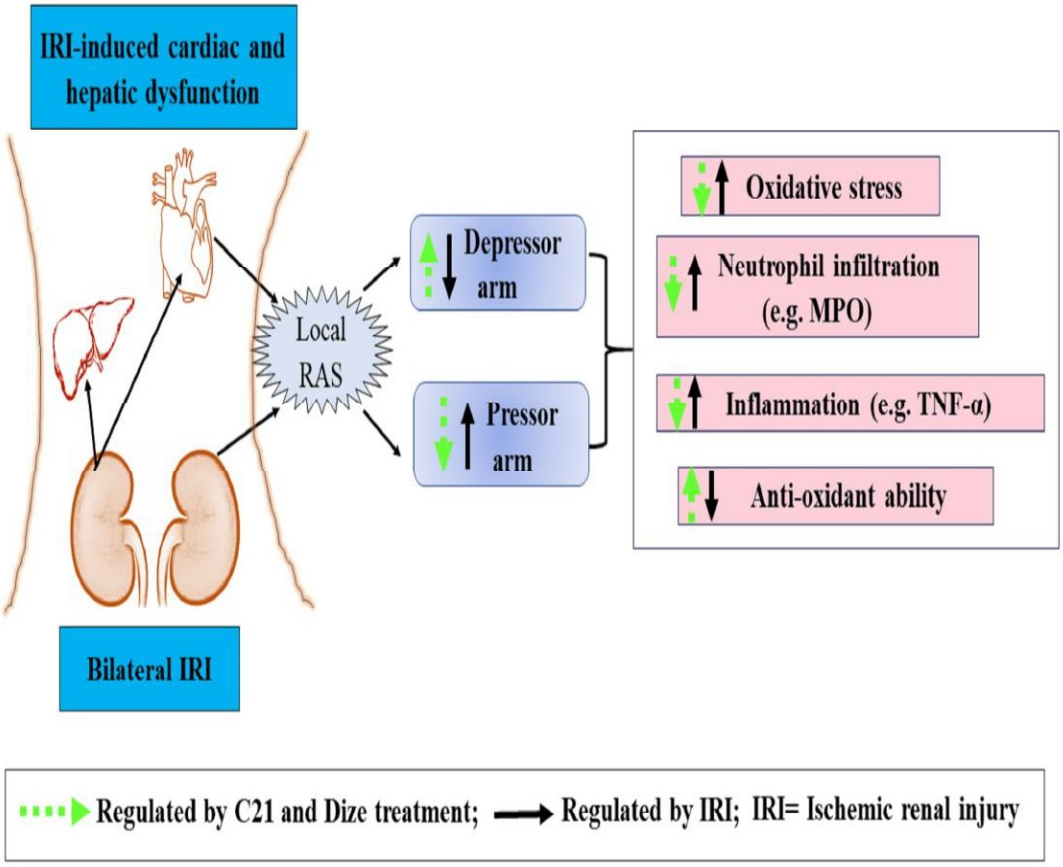

Figure 6.4 AT2R and ACE2 activation prevented IRI-induced cardio-hepatic dysfunction.

IRI activated cardiac and hepatic oxidative stress and inflammatory signaling which was more deteriorated under hyperglycemia-IRI condition. Concomitant administration of C21 and Dize reduced cardiac and hepatic inflammation, attenuated oxidative stress, and improved histological changes in DM and ND rats.

Similarly, we also observed the impact of IRI on cardiac RAS where there was significantly increased in Ang II and ACE levels, Ang (1-7) and ACE2 levels, respectively. In liver homogenates, an analogy result was obtained for the same RAS components. On the contrary, the novel combination therapy managed to normalize the pressor and depressor arm components in ND and DM rats ***(Figure 5.32, 5.33)***. ***Thus, these results support the proposition that depressor arm modulators hold potential for novel therapeutic approaches to curb cardio-hepatic sequelae associated with IRI under diabetic and non-diabetic conditions (Figure 6.4).*** Though the present study has been focused on type 1 diabetes, further studies are needed to evaluate the role of depressor arm of RAS in the progression of AKI and its associated cardiac and hepatic dysfunctions under type 2 diabetic condition as well.