4. Methodology

4.1. Materials

All the instruments and materials used throughout the study were enlisted in the below tables ***(Table 4.1 and Table 4.2)***.

Table 4.1 List of instruments used in the study.

#	Name of the instrument	Make	Country
1.	Invasive blood pressure (IBP) System - PowerLab system - Liquid Pressure Transducer - Animal Bridge Amplifier	AD Instruments	Australia
2.	Microscope (Olympus- BX41) (Zeiss: Vert.A1)	Olympus Zeiss	USA Germany
3.	Laser Doppler	Moor VMF-LDF2	UK
4.	Digital Actophotometer	Inco	India
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.	Tras-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 4.2. Biochemical kits, ELISA kits, and drugs.

#	Name of the product	Suppliers
1.	Streptozotocin	Sigma-Aldrich India (Delhi, India)
2.	Cyproheptadine hydrochloride	Tocris Biosciences, (Bristol, UK)
3.	Diminazene Aceturate	Sigma-Aldrich India (Delhi, India)
4.	Compound 21	Gift sample, Vicore Pharma., Sweden
5.	Biochemical estimation kits for glucose, urea, creatinine, albumin, CK-MB, LDH, ALT, AST.	Accurex Biomedical Pvt. Ltd. (Mumbai, Maharashtra, India).
6.	ACE, ACE2, Angiotensinogen, Ang II, Ang (1-7) and Kim-1 ELISA kits	Wuhan Fine Biological Technology Co., Ltd. (Wuhan, China)
7.	GCSF and GFAP ELISA kits	Elabscience (Wuhan, China)

4.2. Animal studies

All animal studies were done in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Environment, Government of India. These guidelines are according to those of the U.S. Institute of Laboratory Animal Resources (Washington, DC, USA). The animal experiments were carried out at Central Animal Facility (CAF) of Birla Institute of Technology and Science Pilani (BITS-Pilani) as per the protocol approved by the Institutional Animal Ethics Committee (IAEC), BITS-Pilani (Protocol Approval No: IAEC/RES/23/19/Rev-2/25/18). Reported animal studies ensued the ARRIVE guidelines (Kilkenny et al., 2010).

4.2.1. Experimental induction of Type 1 diabetes

The male adult Wistar rats (200-220g) were procured from the CAF of BITS-Pilani and were maintained under standard environmental conditions, with feed and water *ad lib*. Diabetes was induced by injecting a single dose of streptozotocin (STZ) [55 mg/kg, *i.p.*, vehicle- sodium citrate buffer (0.01 M, pH 4.4)] in male Wistar rats, as described previously (Malek & Gaikwad, 2019). ND rats with the same age group received only sodium citrate buffer. After 48 hours of STZ injection, rats showing plasma glucose levels >16 mmol/L were included in the study as DM rats.

4.2.2. Ischemic renal injury in diabetic and non-diabetic rats

ND and DM rats were injected with saline (20 ml/kg, *s.c.*) to prevent fluid loss during laparotomy. Rats were anesthetized with pentobarbital sodium (50 mg/kg, *i.p.*) and kept on a homoeothermic blanket to maintain body temperature (37°C). In studies 1 and 3, unilateral IRI was performed. However, study 2 proceeded with bilateral IRI. After the loss of pedal pain and corneal reflexes, a half-inch incision was given on the left flank portion of the abdomen and the kidney was pulled out of the abdomen by holding the perirenal fat at the lower pole with blunt forceps. Followed by the clamping of renal vascular pedicle with a surgical clamp to induce ischemia (Le Clef et al., 2016) After 45 min and 20 min of unilateral and bilateral ischemia, respectively; the clamps were removed and after observing renal blood flow, the skeletal muscle and skin. Then skeletal muscle and skin layers were sutured separately with absorbable and non-absorbable sutures, respectively. After suturing, topical (Betadine™) antiseptic and parenteral (Augmentin™, 324 mg/kg, *i.p.*) antibiotics were given to prevent post-surgical infection. After Sham control animals were subjected to identical operation without renal vascular pedicle clamping. In unilateral and bilateral ischemia, 48 h and 24 h of reperfusion were performed, respectively.

4.2.3. Study plans and treatment regimens

For study 1:

The first objective of this work was to evaluate the role of epigenetics in the development of acute kidney injury under normal and hyperglycemic conditions. As shown in ***Figure 4.1***, the ND and DM rats were divided into four groups each: (a) **ND/DM-** serve as respective controls, (b) **ND-/DM-IRI-** ND or DM rats subjected to unilateral ischemia (IRI) i.e. 45 min of ischemia followed by 48 h of reperfusion, (c) **ND-/DM-IRI+Cypro-LD-** ND-IRI or DM-IRI rats receiving Cyproheptadine (10mg/kg/day, *p.o.*) (d) **ND-/DM-IRI+Cypro-HD-** ND-IRI or DM-IRI rats receiving Cyproheptadine (20mg/kg/day, *p.o.*) (Murray et al., 2010). All the treatment duration was two days. We kept six rats in each experimental group.

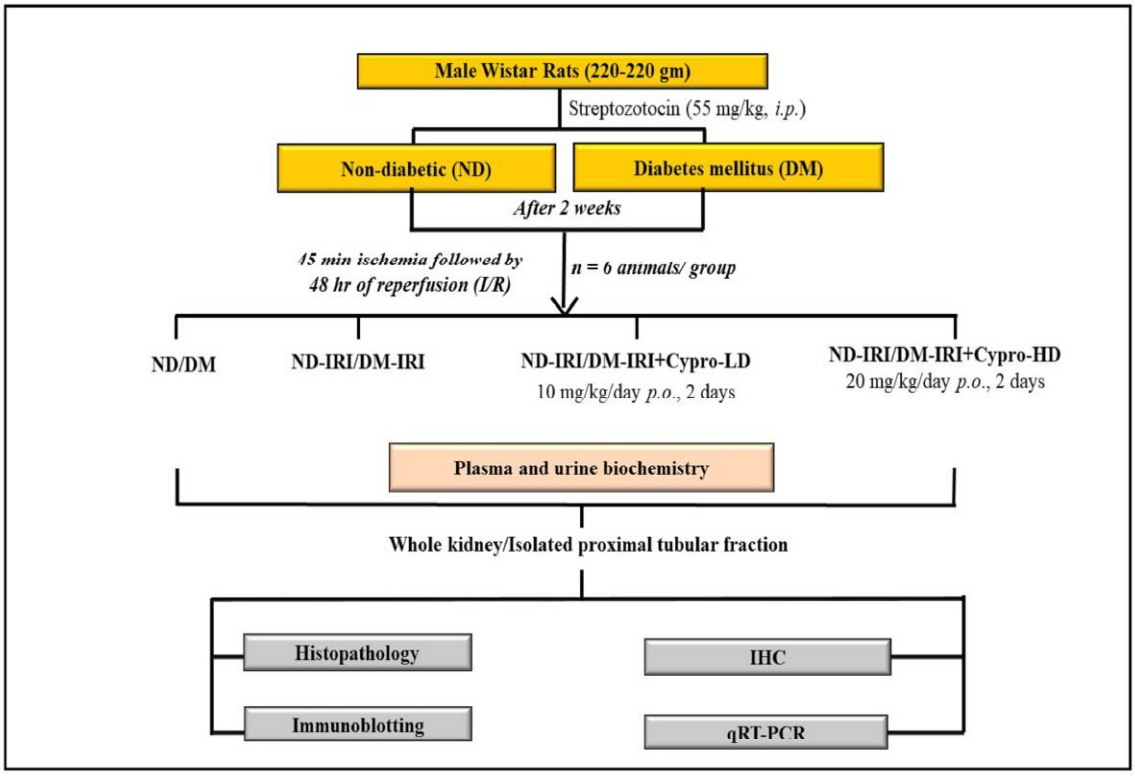

Figure 4.1 A schematic diagram of animal study design 1

For study 2:

The study's second objective was to evaluate the role of AT2 receptor and ACE2 in the development of acute kidney injury under normal and hyperglycemic conditions. As shown in ***(Figure 4.2)***, after two weeks of diabetes induction, both ND and DM rats were subdivided into five groups each: (i) **ND/DM-** serve as respective controls, (ii) **ND-/DM-I/R-** ND or DM rats subjected to Ischemia-45min/reperfusion- 48h (I/R), (iii) **ND-/DM-I/R+C21-** ND-I/R or DM-I/R rats receiving compound 21 (C21) (0.3 mg/kg/day, *i.p.*) (Pandey & Gaikwad, 2017b), (iv) **ND-/DM-I/R+Dize-** ND-I/R or DM-I/R rats receiving Diminazene aceturate (Dize) (5 mg/kg/day, *p.o.*) (Goru et al., 2017b), (v) **ND-/DM-I/R+CD-** ND-I/R or DM-I/R rats receiving C21 (0.3 mg/kg/day, *i.p.*) and Dize (5 mg/kg/day, *p.o.)* combination therapy. All the treatment duration was five days. We kept six rats in each experimental group.

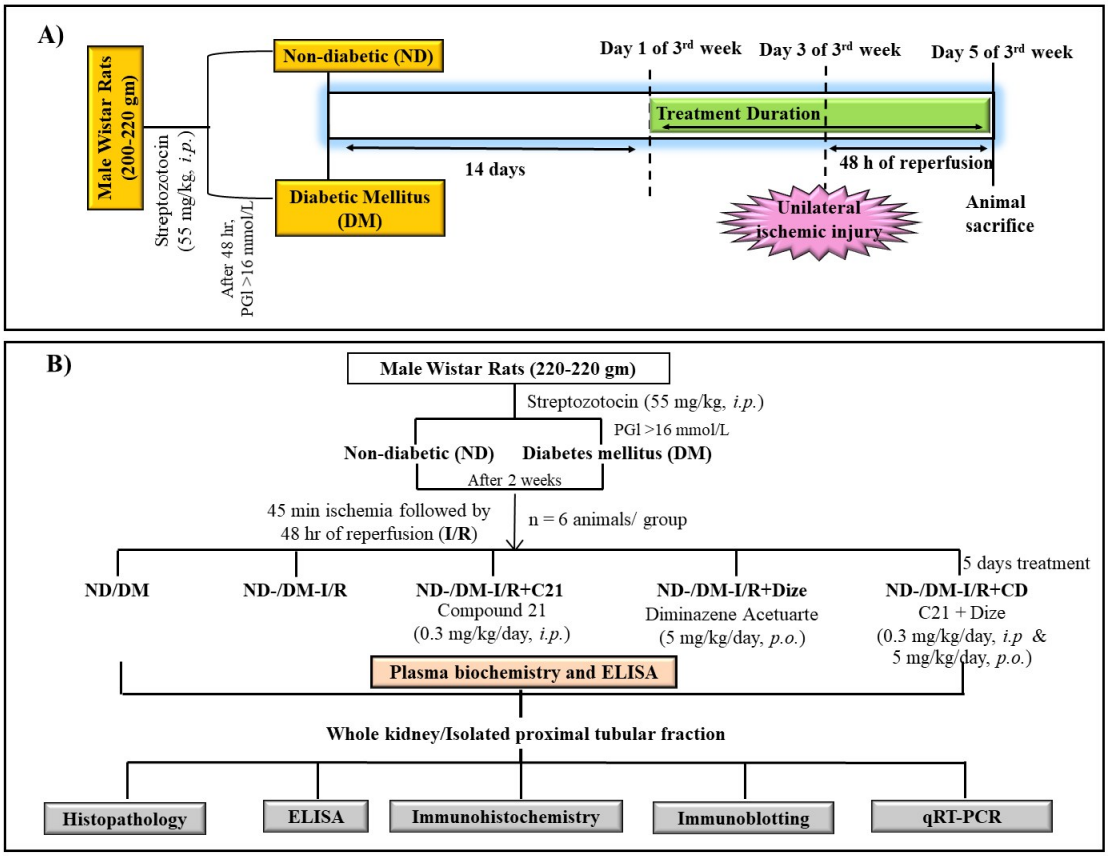

Figure 4.2 A schematic diagram of animal study design 2

For study 3:

The third objective of the work is to study the multiple-organ dysfunction induced by acute kidney injury under normal and hyperglycemic conditions. As shown in ***Figure 4.3***, the ND and DM rats were divided into five groups each: (a) **ND/DM-** serve as respective controls, (b) **ND-/DM-IRI-** ND or DM rats subjected to bilateral ischemia i.e. 20 min of ischemia followed by 24 h of reperfusion, (c) **ND-/DM-IRI+C21-** ND- IRI or DM- IRI rats receiving compound 21 (C21) (0.3 mg/kg/day, *i.p.*) (Pandey & Gaikwad, 2017b), (d) **ND-/DM-IRI+Dize-** ND- IRI or DM- IRI rats receiving Dize (5 mg/kg/day, *p.o.*), (e) **ND-/DM-IRI+CD-** ND- IRI or DM- IRI rats receiving C21 (0.3 mg/kg/day, *i.p.*) and Dize (5 mg/kg/day, *p.o.*) combination therapy. All the treatment duration was four days (three days pre-ischemia and one-day post-ischemia). We kept six rats in each experimental group. At the end of the experiment, the behavioral (locomotor activity) and hemodynamic (invasive blood pressure) experiment were performed.

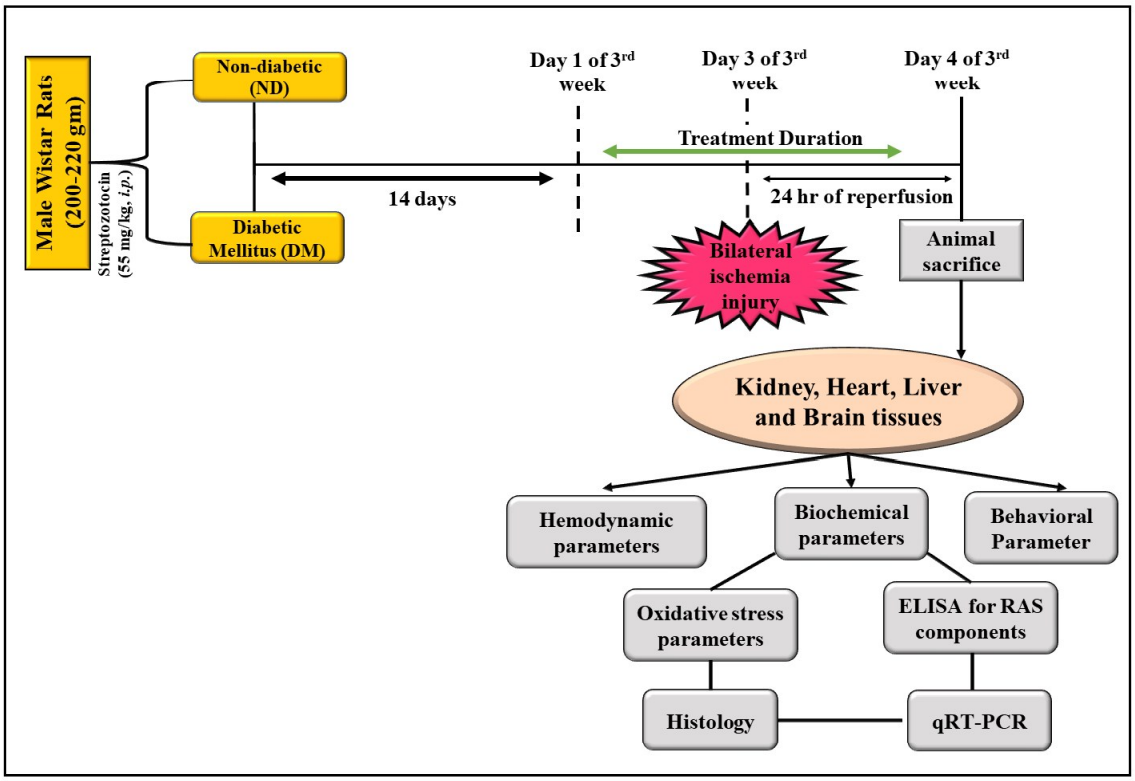

Figure 4.3 A schematic diagram of animal study design 3

4.3. Behavioral parameters

IRI has reported to severely impaired locomotor activity in experimental models. Regarding the mechanism of altered motor activity, increased uremia leads to a change in monoamine metabolism (specifically dopamine) and further altered motor activity (Adachi et al., 2001). Moreover, the connecting link of the reno-cerebral RAS axis remains another possibility to alter motor activity in IRI rats (Cao et al., 2017). In the second objective of the study, we intend to check the role of hyperglycemia and acute kidney injury on neurological dysfunction. Therefore, we performed behavioral assessment using actophotometer (Verma et al., 2018).

4.3.1. Spontaneous locomotor activity

The spontaneous locomotor activity was performed as described by Sharma and Taliyan (Sharma & Taliyan, 2016). After twenty-four hours of renal ischemia, the behavioral assessment was performed between 09:00 and 17:00 h using a digital actophotometer (INCO, India). Briefly, each rat was placed inside a square closed chamber (30×30 cm^2)

and spontaneous locomotor activity for a period of 10 min was recorded using infra-red light-sensitive photocells.

4.4. Assessment of renal functions by plasma and urine biochemistry

After reperfusion time, venous blood (0.5 ml) was collected. Heparin (200 IU mL-1 of blood) was used as an anticoagulant. The plasma was separated by centrifugation (5 min at 5000 g, 4°C). These plasma samples were used for the estimation of plasma glucose (PGL), BUN, and PCr levels using spectrometric kits (Malek et al., 2019b). Moreover, urinary Kim-1 levels were estimated as described by Luo et al, (Luo et al., 2014). After completion of reperfusion time, urine samples were collected using metabolic cages, centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected, aliquoted, and used for estimation of Kim-1 by ELISA.

4.5. Assessment of distant organ functions by plasma biochemistry

Collected plasma samples were assayed for heart-specific [creatine kinase MB (CK-MB) and lactate dehydrogenase (LDH)] and liver-specific [Aspartate aminotransferase (AST) and alanine transaminase (ALT)] functional parameters by using commercially available kits (Accurex) (Malek et al., 2019a; Pandey et al., 2017).

4.6. Systemic blood pressure measurements

Invasive blood pressure (IBP) measurements were taken using an ADInstruments PowerLab system (Bella Vista, NSW, Australia) as described by Malek and Gaikwad (Malek & Gaikwad, 2019). The rats were anesthetized with pentobarbital sodium (50 mg/kg, *i.p.*), and a polyethylene cannula (PE-50, Clay Adams) was introduced into the right carotid artery. The carotid cannula was connected to a liquid pressure transducer (MLT844) attached to the PowerLab signal transduction unit, and blood pressure was taken. After 30 min of the stabilization period, systolic blood pressure (SBP) was recorded for the next 30 minutes.

4.7. Animal sacrifice and organ collection

After completion of IBP measurements, rats were perfused with normal saline (0.9 %W/V NaCl) via a jugular vein cannula followed by sacrification in anesthetized conditions.

Kidney, heart, liver, and brain tissues were collected, washed with ice-cold normal saline and stored into the deep freezer (-80 ºC) to avoid further protein degradation. Further, these tissues were used for molecular biological experiments. For histopathology, the tissues were kept in 10% (v/v) formalin for fixation.

4.8. Proximal tubules isolation from the whole kidney

Collected kidneys were placed in cold PBS (pH- 7.4), and tubular fractions were isolated using the percoll gradient centrifugation method with some modifications (Hakam et al., 2006). 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

Protein content in the heart, brain and liver samples was measured by the Lowry's method using bovine serum albumin [(BSA) 1 mg/ml] as a standard (Classics Lowry et al., 1951). For sample preparation, tissue samples were rinsed with ice-cold isotonic saline (0.9 % w/v NaCl). The heart, brain, and liver tissues were dissected and were homogenized using ice-cold 0.1 M phosphate buffer (pH 7.4) in ten times (w/v) volume, followed by centrifugation at 10,000 g for 15 min (4°C). For proximal tubules, we directly have taken the isolated proximal tubular fraction and added 0.1 M phosphate buffer (pH 7.4) in ten times (w/v) volume. Then, we centrifuged the samples at 10,000 g for 15 min. Finally, the collected supernatant was diluted with 1 ml of distilled water and Lowry's reagent was added. The mixture was thoroughly mixed and kept for 30 min at room temperature. In the last, Folin-Ciocalteu reagent was added followed by 30 min incubation at room temperature. The protein content was measured spectrophotometrically at 660 nm.

4.10. MDA and GSH estimation

The estimation of oxidative stress was carried out by determining the lipid peroxide and reduced glutathione levels in the isolated proximal tubular fraction, heart, liver, and hippocampus. The lipid peroxide level in tissue homogenates was measured as described by Ohkawa et al., 1979. The collected tissue homogenates were centrifuged at 700×*g,* and the supernatant was collected. The absorbance of thiobarbituric acid reacting substances (TBARS) was observed by using the spectrophotometric method at 532nm (Sharma et al., 2015). Then, the concentration of TBARS was assessed from a standard curve and stated as nanomoles per milligram of protein. The GSH content was measured by Ellman's method (Boyne & Ellman, 1972). The homogenized tissue samples were reacted with sulphosalicylic acid and centrifuged. The supernatant was added with Ellman's reagent to develop the yellow color of 5-thio-2-nitrobenzoate-SH. The absorbance was taken at 412 nm.

4.11. Nitrite levels estimation

Nitrite, an indicator of nitric oxide production, was done as described by Sharma, S., et al., (Sharma et al., 2015). Briefly, nitrite levels were determined by Griess reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1 % sulfanilamide, and 2.5 % phosphoric acid). Equal volumes of Griess reagent and tissue (proximal tubules/hippocampus) supernatant were mixed together and were incubated in the dark light for 10 min (24℃). Absorbance was taken at 540 nm and the concentration of nitrite was assessed from a sodium nitrite standard curve and expressed as micromoles per milligrams protein.

4.12. Myeloperoxidase Activity

Myeloperoxidase (MPO) activity in the heart and liver tissues of rats was measured as described previously (Yang et al., 2017c). Briefly, aliquots of supernatants were analyzed using *o*-dianisidine dihydrochloride, 0.0005% hydrogen peroxide and 0.1 mM H_2O_2, and incubated to 37°C. MPO activity was determined at 450nm using a spectrophotometer, further normalized to protein content and expressed as U/mg protein.

4.13. Histology

Histology was performed as per the protocol explained by Pandey et al., (Pandey et al., 2015). Briefly, the kidney, heart, liver, and brain tissues were 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%, 95%, 70%, 50%; 3 minutes each). Hematoxylin and eosin staining were performed as described previously (Danelli 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 400× and 100x magnification by using a Zeiss microscope (model: Vert.A1) and Optika TCB5" microscope (Optika Research Microscope, Italy), respectively.

4.14. Immunohistochemistry

Immunohistochemistry was performed as described previously (He et al., 2019; Karpe & Tikoo, 2014). Briefly; the kidney, heart, liver, and brain 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. The list of primary and secondary antibodies used has been mentioned in ***Table 4.3.***

4.15. Cytoplasmic and histone protein isolation

Cytoplasmic and histone proteins were isolated from proximal tubular fraction as previously described protocols (Malek & Gaikwad, 2019; Tikoo et al., 2008). For cytoplasmic protein separation, isolated proximal tubular pellet was mixed with ice-cold PBS, and homogenized (1000 rpm for 30 sec) in the low salt buffer (LSB) by using a homogenizing probe. LSB comprises 150 mM EDTA, 10 mM Tris-HCl; pH 7.4, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulphonyl fluoride, 1 mM sodium butyrate and protease inhibitor cocktail. The lysates were then centrifuged at 12,000 g at 4°C for 7 min. The protein concentration was estimated by Lowry's method.

For histone extraction, 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% SDS and the desired protein was resolved according to molecular weight. 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. The primary and secondary antibodies utilized are listed in ***Table 4.3***.

Table 4.3 List of antibodies used throughout the study

#	Antibody Name	Dilution	Company
1.	***Primary antibody against:*** p-NFκ-β(S-536) (#3033) IκBα (#4814) cleaved PARP (#5625) cleaved Caspase-3 (#9664) SET7/9 (#2813) Symd2 (#9734) RBBP5 (#13171) H3K36Me2 (#2901) H3K9Me2 (#4658) H3K4Me2 (#9725) H3 (#4499)	1:1000 (v/v)	Cell Signaling Technology (Danvers, MA, USA)
2.	***Primary antibody against:*** MCP-1 (sc-1785) β-actin (sc-4778)	1:1000 (v/v)	Santa Cruz Biotechnology (Dallas, Texas, USA)
3.	***Secondary antibodies:*** Goat Anti-rabbit IgG (#7074) Horse anti-mouse IgG (#7076)	1:20000 (v/v)	Cell Signaling Technology (Danvers, MA, USA)
4.	***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)

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

RNA was isolated from proximal tubules and hippocampus by using TRIzol reagents and purified by Ambion™ PureLink™ RNA Mini Kit (Thermo Fisher Scientific, MA, USA).

qRT-PCR was performed using specific primers ***(Table 4.4)***, designed and produced by Eurofins, India. Briefly, 5 µg of RNA was taken and incubated with 1 µl (2U) of recombinant DNase1 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 DNaseI. 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 & Gaikwad, 2019).

Table 4.4 List of primer sequences utilized for qRT-PCR.

Gene Name	**Primer sequences of qRT-PCR**
At1r	Forward 5'-CTCTGCCACATTCCCTGAGTT-3' Reverse 5'-CTTGGGGCAGTCATCTTGGA-3'
At2r	Forward 5'-GCCCTAAAAAGGTGTCCAGCA-3' Reverse 5'-CAGGTCCAAAGAGCCAGTCA-3'
Masr	Forward 5'-AAGACCAGCCCACAGTTA-3' Reverse 5'-TCGATCACAGGAAGAGAG-3'
Il6	Forward:5'-GGATACCACCCACAACAGAC-3' Reverse:5'-GAAACGGAACTCCAGAAGAC-3'
Tnfa	Forward:5'-GATCGGTCCCAACAAGGAGG-3' Reverse:5'-CTTGGTGGTTTGCTACGACG-3'
Mcp1	Forward 5'-GTCTCAGCCAGATGCAGTTA-3' Reverse 5'- CCTTATTGGGGTCAGCACAG-3'

Nfkb p65	Forward:5′-CATCACACGGAGGGCTTC-3 Reverse:5′-GAACGATAACCTTTGCAGGC-3′
Set7/9	Forward 5'-AGGTTGACAGCAGGGATT-3' Reverse 5'-CAGTTCGGAGAAGGGAGT-3'

4.17. Statistical analysis

Experimental values are represented as mean ± S.D. and n refers to the number of samples studied. Statistical comparison between two groups was performed by using t-test. Moreover, statistical comparison in more than two groups was performed using two-way analysis of variance (ANOVA) and 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 7.00 (San Diego, CA, USA). Data for which $p < 0.05$, were considered statistically significant.