CHAPTER-1

Ameliorative effect of telmisartan, azilsartan and ramipril in streptozotocine induced diabetic neuropathy and nephropathy in rats: Possible behavioural, biochemical, electrophysiological and neuroinflammatory evidences

1.1. Introduction

Diabetes is a chronic metabolic disorder causing significant health burden on worldwide population. According to International Diabetes Federation, number of the diabetic patients would increase to 592 million by the year of 2035 (Guariguata et al. 2014). Diabetes is associated with various microvascular complications that include diabetic neuropathy, nephropathy and retinopathy. Burden of these complications are increasing due to inadequate therapeutic treatment to control and manage this disease (Mehra et al. 2014). In India, type 2 diabetic patients are near to 62.4 million and 77 million with pre-diabetes (Anjana et al. 2011). These numbers have been projected to climb to 101 million by 2030 (Dasgupta 2014). Among these, the prevalence of diabetic neuropathy and diabetic nephropathy were around 26.1%, 26.9% respectively (Rani et al. 2010; Mohan and Anbalagan 2013). The prevalence of neuropathic pain is also likely to increase in coming future due to the higher survival rates from diseases that are associated with neuropathic pain, such as diabetes, cancer and HIV infection (Davis et al. 2011; Schaefer et al. 2014).

Chronic hyperglycaemia is the major culprit that ultimately causes metabolic and structural abnormalities including but not limited to various classical metabolic pathways like AGEs, PKC, aldose reductase, hexosamine pathways (Brownlee 2005). Further, clinical trials targeting these pathways are not very much encouraging and so far only pregabalin, duloxetine have been approved by USFDA for diabetic neuropathy (Schreiber et al. 2015). This emphasized the need of newer therapies or approaches to target diabetic complications. However, strict control of blood glucose is the prime target for the control of disease progression. Neuroinflammatory, oxidative stress have been well documented in the diabetes and agents directed towards these pathways have
been shown to have promising results in recent study (Sandireddy et al. 2014; Kumar and Sharma 2010; Shi et al. 2013; Drel et al. 2010).

Increased levels of angiotensin-II with hyperglycemia and alteration of RAS has produced a dramatic impact on disease progression in diabetic patients (Nickenig 2002). Moreover, diabetic neuropathy and nephropathy have been associated, apart from the activation of above mentioned classical metabolic pathways (increased polyol pathway, hexosamine flux, AGEs, PKC activation) with the alteration in vascular reactivity, increase in oxidative/nitrosative stress, immune and inflammatory components leading to neuronal cell death (Sandireddy et al. 2014; Kumar et al. 2012).

ROS production by NAD(P)H oxidase through angiotensin-II and also by mitochondrial electron transport chain (ETC) dysfunction ultimately lay as a central platform for the above mentioned cascade of events in diabetes (Chen et al. 2009; Sedeek et al. 2013). Further, hyperglycemia is well known to alter directly ETC functioning leading to an increased production of ROS and reduced adenosine tri-phosphate (ATP) production. Besides, increased ROS are known to cause direct DNA damage that lead to cell death (Rolo and Palmeira 2006). So, ability to reduce ROS generation has been proposed to be as one of the effective strategies to prevent the neuronal damage with free radical scavengers and or antioxidant agents (Oyenihi et al. 2015; Matough et al. 2012).

Besides, accumulation of advanced glycation end (AGE) products results in activation of inflammatory and immune pathways by acting on receptors present on glial and macrophages (Gkogkolou and Bohm 2012). Activation of glial cells and macrophages leads to production of different inflammatory mediators like TNF-α, IL-1, IL-6, chemo attractant protein-1 etc. Further, activation NF-kB pathway has been well known and this will further accelerate inflammatory cascade via positive feedback mechanism (Singh et al. 2014; Wada and Yagihashi 2005). So, targeting these inflammatory pathways could be a promising approach in treating diabetic complications and several other agents directed towards these inflammatory pathways have been shown to produce promising results (Shi et al. 2013).
Traditionally, ARBs & ACE-inhibitors are known as first line agents for their antihypertensive (Li et al. 2014), cardioprotective (Munger 2011), renoprotective actions (Baltatzi et al. 2011). ARBs act mainly through AT1 receptor blockade. However, their neuroprotective actions have also been claimed because of their additional antioxidant, neuroinflammatory and antiapoptotic actions in different neurodegenerative conditions which are independent of their antihypertensive actions (Garrido-Gil et al. 2012; Pang et al. 2012; Dorenkamp et al. 2005). However, their potential role in diabetic neuropathy and nephropathy is still sparse and not fully explored.

Various ARBs and ACE-I have been well suggested to improve vascular and neuronal functions in diabetic patients of which telmisartan has well reported to have neuroprotective action. Similarly, azilsartan is newly developed ARB and has been shown to exert potent antihypertensive effects in various randomized control trials (Malik et al. 1998; Takagi et al. 2014; White et al. 2011). There is ample evidence available for angiotensin-II to participate in nociceptive processing at central and peripheral sensory systems (Pelegrini-da-Silva et al. 2005; Marques-Lopes et al. 2009; Pavel et al. 2008; Coppey et al. 2006). Angiotensin-II has been produced not only by circulating but also by tissue specific RAS, shown to participate in nociceptive processing in brain and spinal cord apart from having its effects on sympathetic and hormonal control (Bader and Ganten 2008; Patil et al. 2010). Recently, the presence of angiotensin-II and its co-localization with CGRP & SP in the DRG neurons has been demonstrated for nociception (Patil et al. 2010). Indeed, angiotensin-II mediated injury of primary afferent neurons and glial cell activation in nerve injured rats demonstrates the potential of AT1 receptor participation in chronic pain (Pavel et al. 2013). Further, renin inhibitors, angiotensin and aldosterone antagonists have been shown to produce beneficial effects in different types of nerve injury models (Jaggi and Singh 2011; Khan et al. 2009).

Telmisartan has been reported to produce neuroprotective effects via AT1 and PPARγ receptors in different neurodegeneration conditions apart from its renoprotective effect (Wang et al. 2014; Garrido-Gil et al. 2012; Thoene-Reineke et al. 2011). Azilsartan is a potent blood pressure lowering agent among ARBs, currently available with potent insulin sensitizing actions demonstrated in rat and
dog models (Kusumoto et al. 2011; Zhao et al. 2011). Further, it has been shown to exert antiinflammatory effects (Matsumoto et al. 2014) and modulate PPARγ receptors (Iwai et al. 2007).

Ramipril, a potent ACE-I and known to posses antioxidant properties (El Midaoui et al. 2008). It has been shown to possess antiinflammatory, antioxidant properties and shown neuroprotective effect in different disease conditions like cerebral ischemia (Yusuf et al. 2000), cardiac fibrosis (Shi et al. 2012), glutamate induced neurotoxicity (Sengul et al. 2011), ROS induced white matter lesions (Kim et al. 2008) but data showing its effect in diabetic neuropathy and nephropathy is very sparse and needs further investigations.

Utilizing this knowledge, the present research work has been designed to investigate the possible protective effects of telmisartan, azilsartan & ramipril in streptozotocine induced diabetic neuropathy and nephropathy with the help of behavioral, biochemical, cellular and electrophysiological parameters.

1.2. Materials and Methods

1.2.1. Animal husbandry

The Wistar male rats (200-250 g) obtained from central animal house (CAH), Panjab University (Chandigarh) were utilised in the current study. The animals were kept on regular 12-h light/dark cycle with free access to food and water and. All the experiments were performed between 9:00 and 17:00 h. Institutional Animal Ethics Committee, Panjab University approved the experimental protocol (IAEC/346-56/UIPS, 11/02/2013) and experiments were conducted according to the Indian National Science Academy guidelines for the use and care of experimental animals.

1.2.2. Induction and assessment of diabetes

Streptozotocine (STZ) was solubilized in 0.1M citrate buffer (pH 4.5) & administered in single intraperitoneal (i.p.) injection to overnight fasted rats. Glucose (5% w/v) supplementation was given for first 8 hr after STZ injection to prevent hypoglycaemic shock. After 48hrs of STZ injection, blood samples were taken from retro-orbital plexus under light ether anaesthesia.
1.2.3. Drug and treatment schedule

Whole study protocol (Fig. 1.1) consists of nine treatment groups (n = 6-8 in each group) (Table 1.1). Telmisartan, azilsartan, ramipril were supplied by IPCA (Mumbai). STZ, was purchased from Sigma-Aldrich (USA), India. STZ was dissolved in 0.1M citrate buffer (pH4.5) and all other drugs were suspended in 0.25% (w/v) carboxy methyl cellulose (CMC) solution. Telmisartan, azilsartan, ramipril were administered orally (p.o.), once daily in diabetic animals (blood glucose > 250mg/dl) and continued for 8 weeks. All the drugs were administered, daily in the morning 10: 00 h. Knowledge of previous reports was utilized in determining the drug doses (Xu and Liu 2013; Zhao et al. 2011; Amann et al. 2000).

1.2.4. Behavioural Assessments

1.2.4.1. Body weight

Body weights of all the animals were documented on day 0 prior to STZ injection and on day 56. Difference in body weight on day 0 and day 56 of the study was computed according to the following formula:

Percentage change in body weight =

\[
\frac{\text{[Body weight (day 56 - day 0)]} \times 100}{\text{(Body weight on day 0)}}
\]

1.2.4.2. Assessment of food and water intake

Food and water intake of each individual group of animals were recorded on the last day of the study.
Table 1.1 Treatment groups

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Treatment group</th>
<th>Treatment (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal control</td>
<td>Healthy animals (No treatment given)</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle control</td>
<td>Healthy animals (Vehicle administered)</td>
</tr>
<tr>
<td>3</td>
<td>Diabetic control</td>
<td>Single i.p injection of STZ (55 mg/kg) administration, dissolved in 0.1 M citrate buffer (pH 4.5).</td>
</tr>
<tr>
<td>4</td>
<td>STZ+T(5)</td>
<td>Telmisartan (5 mg/kg, p.o.) administered to single i.p STZ (55 mg/kg) administered animals</td>
</tr>
<tr>
<td>5</td>
<td>STZ+T(10)</td>
<td>Telmisartan (10 mg/kg, p.o.) administered to single i.p STZ (55 mg/kg) administered animals</td>
</tr>
<tr>
<td>6</td>
<td>STZ+A(2)</td>
<td>Azilsartan (2 mg/kg, p.o.) administered to single i.p STZ (55 mg/kg) administered animals</td>
</tr>
<tr>
<td>7</td>
<td>STZ+A(4)</td>
<td>Azilsartan (4 mg/kg, p.o.) administered to single i.p STZ (55 mg/kg) administered animals</td>
</tr>
<tr>
<td>8</td>
<td>STZ+R(0.2)</td>
<td>Ramipril (0.2 mg/kg, p.o.) administered to single i.p STZ (55 mg/kg) administered animals</td>
</tr>
<tr>
<td>9</td>
<td>STZ+R(2.3)</td>
<td>Ramipril (2.3 mg/kg, p.o.) administered to single i.p STZ (55 mg/kg) administered animals</td>
</tr>
</tbody>
</table>

1.2.4.3. Assessment of mechanical allodynia

Animals were transferred individually on to an elevated mesh (1 cm² perforations) in a clear plastic cage and adapted to the testing environment 30 min prior to the actual test. Mechanical allodynic response was quantified by using vonfrey anesthesiometer and rigid vonfrey filaments. A polypropylene rigid tip (0.5 mm diameter) was used to apply the force to plantar region of the hind paw. The force that leads to paw withdrawal was recorded by the vonfrey anesthesiometer. The test was repeated 3 times with a 5min intervals and the mean value was recorded (Negi et al. 2010).
1.2.4.4. Assessment of mechanical hyperalgesia

Paw pressure thresholds were registered with Randall-Selitto analgesia meter (IITC Life Science, Woodland Hills, CA). Increasing pressure at a linear rate of 10 gm/sec was applied to the center of the hind paw. Pressure, at which the animal exhibited pain by withdrawal of the paw, was registered by an analgesia meter and expressed in mass units (grams), with a cut-off of 250g to avoid potential tissue injury. Three test recordings separated by at least 10 min intervals were performed for each animal, and mean value of these tests was expressed (Santos-Nogueira et al. 2012).

1.2.4.5. Assessment of thermal hyperalgesia

Both peripheral & central machinery are said to be involved in hyperalgesic response through hot-plate. In this test, animals were placed individually on Eddy's hot plate maintained at 52 ± 1°C. The time taken for the first sign of paw licking or jumping response indicates the pain threshold. 15 sec cut-off time was used to avoid paw damage (Choi et al. 1994).

1.2.5. Blood collection and processing

Following behavioral assessments, blood samples were collected from individual animals from retro-orbital route; centrifuged at 5000 rpm for 5 minutes, serum was collected & stored at -20°C. Overnight urine was collected using metabolic cages on day before the completion of protocol, at -20°C until analysis.

1.2.5.1. Assessment of serum and urine parameters

1.2.5.1.1. Assessment of serum glucose

Blood glucose levels were assayed by following the GOD-POD method using the glucose estimation kit (AMS analysers, Italy).

Principle- By the action of glucose oxidase (GOD) glucose is converted in to gluconic acid & H₂O₂. This H₂O₂ reacts with phenol & 4-aminpantypirine in presence of peroxidase (POD), leading to a red complex formation. The color intensity of this complex at 505 nm is documented which proportional to the quantity of glucose in the test sample.

\[
\text{GOD} \quad \text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{Gluconic acid} + \text{H}_2\text{O}_2
\]
POD
H₂O₂ + Phenol + 4-Aminoantipirine \rightarrow \text{Red complex + H}_2\text{O}

Composition

Reagent A:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer pH 7.4</td>
<td>25 g/l</td>
</tr>
<tr>
<td>Phenol</td>
<td>&lt; 0.9 g/l</td>
</tr>
<tr>
<td>4-Aminoantipirine</td>
<td>0.4 mmol/l</td>
</tr>
<tr>
<td>GOD</td>
<td>\geq 30 kU/l</td>
</tr>
<tr>
<td>POD</td>
<td>\geq 1 kU/l</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.95 g/l</td>
</tr>
</tbody>
</table>

Standard:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>100 mg/dl (5.55 mmol/l)</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>&lt; 14.7 mmol/l</td>
</tr>
</tbody>
</table>

Assay procedure

1. 1000 μL of Reagent A was added to each test tube.
2. 10 μL of standard or sample or distilled water were then added to respective test tubes to make standard, sample and blank solutions respectively. The tubes were mixed well and incubated at 37°C for 10 minutes.
3. Blank solution was used to set auto zero of the spectrophotometer and the absorbance were read at 505 nm.

Calculation

Results of serum glucose was quantified using

\[ \text{Serum Glucose} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100 \]

1.2.5.1.2. Assessment of serum/urinary creatinine

Serum/urinary creatinine levels were assayed by following the reaction with picric acid using the creatinine estimation kit (AMS analysers, Italy)

Principle- Creatinine present in test samples reacts with picric acid in alkali giving rise to a yellow-orange chromophore. The intensity of the color is indicative of the amount of creatinine present in the sample.
Composition

Reagent A:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium hydroxide</td>
<td>1.25 mmol/l</td>
</tr>
</tbody>
</table>

Reagent B:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Picric acid</td>
<td>20.5 mmol/l</td>
</tr>
</tbody>
</table>

Assay procedure

1. 100 μL of sample (serum/urine) was added to 500 μL Reagent A in clean and dry test tube, mix well and incubated for 5min.
2. Then 500 μL of Reagent B was added to the above reaction mixture.
3. Mixed and after 10 seconds absorbance (A1) was read at 490nm and again after 1 min absorbance (A2) was read.

Calculation

Change in absorbance (ΔA) was calculated as ΔA= (A2-A1), for all the samples and standard.

Results of creatinine was calculated using the following formula

\[
\text{Serum Creatinine (mg/dL)} = \frac{\Delta A \text{ of sample}}{\Delta A \text{ of standard}} \times 2
\]

\[
\text{Urine Creatinine (mg/dL)} = \frac{\Delta A \text{ of sample}}{\Delta A \text{ of standard}} \times 50
\]

1.2.5.1.3. Assessment of serum urea nitrogen

Serum/urinary urea and nitrogen levels were assayed by following the urease method using the urea estimation kit (AMS analysers, Italy).

Principle- Urea is hydrolyzed by the action of urease enzyme resulting in formation of ammonia and carbon dioxide. The formed ammonia reacts with 2-ketoglutarate by the action of glutamate dehydrogenase with simultaneous oxidation of NADH into NAD+.
Urease
Urea + 2 H₂O → 2 NH₄⁺ + 2 HCO₃⁻

GLDH
2-Ketoglutarate + NH₄⁺ + NADH → L-Glutamate + NAD⁺ + H₂O

The reduction in NADH absorbance at 340 nm, is directly related to the amount of urea present in the sample.

Composition

Reagent A:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS pH 7.8</td>
<td>150 mmol/l</td>
</tr>
<tr>
<td>2-Ketoglutarate</td>
<td>8.75 mmol/l</td>
</tr>
<tr>
<td>ADP</td>
<td>0.75 mmol/l</td>
</tr>
<tr>
<td>Urease</td>
<td>≥7.5 kU/l</td>
</tr>
<tr>
<td>GLDH (Glutamate-dehydrogenase)</td>
<td>≥1.25 kU/l</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>≤0.95 g/l</td>
</tr>
</tbody>
</table>

Reagent B:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>1.32 mmol/l</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>≤0.95 g/l</td>
</tr>
</tbody>
</table>

Standard:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>50 mg/dl</td>
</tr>
</tbody>
</table>

Assay procedure

1. 10 μL of sample (serum/urine)/standard/distilled water was added to 800 μL Reagent A in each clean and dry test tubes separately, mix well and incubated for 5 min at 37°C.

2. Then 200 μL of Reagent B was added to the above reaction mixture.

3. The above reaction mixture was mixed well and incubated for 30 seconds at 37°C, then absorbance (A1) was read at 340 nm for the blank, standard and sample and precisely after 1 min absorbance (A2) was read.

Calculation

Change in absorbance (ΔA) was calculated as
ΔA = [(A1-A2) sample or standard] – [(A1-A2) blank], for all the test and standard solutions.

Results of serum/urine urea and nitrogen were calculated using the following formula

\[
\text{Serum Urea (mg/dl)} = \frac{\Delta A_{\text{Sample}}}{\Delta A_{\text{Standard}}} \times 50
\]

\[
\text{Urine Urea (g/24h)} = \frac{\Delta A_{\text{Sample}}}{\Delta A_{\text{Standard}}} \times 10 \times \text{urine output in 24hrs}
\]

Conversion factor for Blood Urea Nitrogen (BUN) calculation

Urea (mg/dl) × 0.467 = BUN (mg/dl).

1.2.5.1.4. Assessment of total proteins

Principle- This test is based on the formation of red color product upon reaction of proteins with copper ions in alkali. Color intensity is proportional to proteins concentration. Red color is overlapped with light blue colour of the reagent leading to the violet colour at the end.

Composition

Reagent A (liquid)

<table>
<thead>
<tr>
<th>Composition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-Na Tartrate</td>
<td>318 mmol/l</td>
</tr>
<tr>
<td>KJ</td>
<td>30 mmol/l</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>12 mmol/l</td>
</tr>
<tr>
<td>NaOH</td>
<td>600 mmol/l</td>
</tr>
<tr>
<td>Corrosive</td>
<td>R34; S(1/2)26-37/39-45</td>
</tr>
</tbody>
</table>

Standard (liquid)

| Stabilized proteic solution | 6 g/dl |

Assay procedure

1. 20 μL of sample / standard was added to 1000 μL Reagent A in each clean and dry test tube separately, mixed well and incubated for 10 min at room temperature.
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2. The absorbance (A) of the test sample and standard samples were read at 546nm against the blank.

Calculation

\[
\text{Total proteins (g/dl)} = \frac{A \text{ sample}}{A \text{ standard}} \times 6
\]

1.2.5.1.5. Assessment of serum albumin

The serum albumin content is determined using its reaction with bromocresol green (BCG) reagent.

Principle- Albumin reacts with BCG in the presence of citrate buffer to form a colored compound. Intensity of the color is direct indicative of the albumin concentration present in the sample.

Composition

Reagent A

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate buffer</td>
<td>7.5 mmol/l</td>
</tr>
<tr>
<td>BCG</td>
<td>≥ 150 µmol/l</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.05%</td>
</tr>
</tbody>
</table>

Standard (liquid)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>4 g/dl</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.05%</td>
</tr>
</tbody>
</table>

Assay procedure

1. 5 µL of sample / standard was added to 1000 µL Reagent A in each clean and dry test tube separately, mixed well and incubated for 5 min at room temperature.

2. The absorbance (A) of the test solutions & standards was read at 546nm against the blank.

Calculation

\[
\text{Serum albumin (g/dl)} = \frac{A \text{ sample}}{A \text{ standard}} \times 4
\]
1.2.6. Assessment of motor nerve conduction velocity (MNCV)

MNCV was recorded using Power Lab 26T (AD Instruments, Australia) system according to the method of (Saini et al. 2004) with slight modifications. Briefly, animals were anaesthetised with thiopentone sodium (30mg/kg, i.p), and core temperature was maintained at 37 ± 1 °C. Recording electrodes were placed superficially in to the plantar region of the foot and sciatic nerve was stimulated with 3V proximally at sciatic notch and distally at tibial notch. The latencies for the compound muscle action potentials were recorded using bipolar needle electrodes was measured from the stimulus artefact to onset of negative M-wave deflection. MNCV was calculated as follows

\[
\text{MNCV} = \frac{\text{Distance between sciatic and tibial stimulation points}}{\text{(Sciatic M wave latency - Tibial M wave latency)}}
\]

1.2.7. Dissection and homogenization

Cervical dislocation is employed to sacrifice all the animals. Both sciatic nerves and kidneys were rapidly collected and placed on ice. For biochemical analysis, tissues were individually homogenised in 0.1 M phosphate buffer (pH 7.4) to get a 10% (w/v) homogenate for sciatic nerve and kidney. The homogenates were centrifuged at 10,000 × g at 4 °C for 15 min. Supernatants produced were separated and used for biochemical estimations with the help of Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, USA).

1.2.7.1. Measurement of endogenous antioxidant profile

1.2.7.1.1. Measurement of lipid peroxidation

Lipid peroxidation (LPO) is the oxidative degradation of lipids leading to formation of reactive malondialdehyde (MDA). Measuring these end products is the common methodology used to assess oxidative stress. MDA reacts with thiobarbituric acid (TBA) leading to formation of a chromophore adduct (MDA-TBA) and can be quantified colorimetrically. The quantitative measurement of LPO was carried out as described by Wills (Wills 1966). Briefly, the MDA present in test sample was quantified by its reaction with TBA at 532 nm using Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, USA). Molar extinction
coefficient of chromophore \((1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1})\) was used to calculate the results.

1.2.7.1.2. *Reduced glutathione (GSH) assay*

GSH was estimated as described by Ellman (Ellman 1959). 1.0 ml of test homogenate and sulfosalicylic acid (4%) were mixed, incubated at \(4^\circ\text{C}\) for 1 h duration & centrifuged immediately at 1200 \(\times\) g for 15 min at \(4^\circ\text{C}\). Supernatant (0.1 ml) thus obtained is mixed with 2.7 ml of phosphate buffer (pH 8.0) & 0.2 ml of 0.01 M di-thio, bis-nitro benzoic acid (DTNB). Absorbance of the yellow chromophore was read at 412 nm, immediately using Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, USA). Results are computed using molar extinction coefficient of chromophore \((1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1})\) & expressed in nanomole GSH per milligram protein.

1.2.7.1.3. *Superoxide dismutase (SOD) activity*

SOD mediated inhibition of the reduction of nitro blue tetrazolium (NBT) was the basis for this assay. Briefly, to the mixture of test homogenate, and NBT, hydroxylamine hydrochloride was added and the absorbance was read at 560 nm (Kono 1978). The results were presented as unit/milligram protein. One unit of enzyme refers to the quantity of enzyme required to inhibit the reaction rate by 100%.

1.2.7.1.4. *Catalase estimation*

Catalase activity was assayed by the method of Luck, wherein breakdown of \(\text{H}_2\text{O}_2\) is measured at 240 nm. Briefly, the reaction mixture consists of 3 ml of \(\text{H}_2\text{O}_2\), phosphate buffer & 0.05 ml of supernatant and then the change in absorbance at 240 nm was documented with a Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, USA). The results were expressed as micromole \(\text{H}_2\text{O}_2\) decomposed per milligram of protein/min (Luck 1965).

1.2.7.1.5. *Protein estimation*

Protein estimation was done by biuret method using bovine serum albumin as standard (Gornall et al. 1949).

1.2.7.2. *Estimation of tumour necrosis factor-alpha (TNF-\(\alpha\)) in sciatic nerve and kidney*
TNF-α has been well known to take part in normal host immune and inflammatory response. Its level in sciatic nerve and kidney was quantified by using rat TNF-α kit (BD Biosciences, San Jose, CA, USA). It is a sandwich enzyme linked immunosorbent assay (ELISA), which is quantified at 450 nm with ELISA reader. TNF-α concentration in test samples was calculated from standard curves.

Principle- This assay employs the quantitative sandwich ELISA technique. A purified monoclonal antibody specific for rat TNF-α has been pre-coated on a 96 well plate. Standards and test samples are added into the wells and any rat TNF-α present would bind with the immobilized antibody. Unbound substances were washed, and then biotinylated anti rat-TNF antibody is added. Following a second wash, streptavidin-horseradish peroxidase conjugate is added to the wells. Again after a third wash, TMB substrate solution is added giving rise to blue product that turns yellow upon the addition of stop solution. The colour intensity is directly proportional to the the amount of rat TNF-α bound in the initial step.

**Assay procedure**

1. All the dilutions were prepared as per the manufacturer's instructions.
2. 50 μL of ELISA diluent was added to each well of the pre-coated 96 well plate.
3. 50 μL of standard or test sample was then added to each well and were mixed gently. The plate was covered with the adhesive strip & incubated for 2 hours at room temperature.
4. Each well was then aspirated and washed with wash buffer for 5 times.
5. 100 μL of rat TNF-antibody was added to each well and covered with a new adhesive strip. The plate was again incubated for 1 hour at room temperature.
6. Aspiration/washes were repeated as in step 4.
7. 100 μL of enzyme working reagent was then added to each well & again incubated at room temperature for 30 minutes.
8. Aspirated/washed as in step 4 for 7 times.
9. 100 μL of TMB substrate solution was added to each well and incubated for 30 minutes at room temperature in dark.
10. Finally, 50 \( \mu \)L of stop solution was added to each well and the absorbance of all the wells was read in an ELISA reader (BioRad, X-merk) at 450 nm.

**Calculations**

The average for the duplicate readings was calculated for each standard and sample and the blank values were subtracted. A standard curve was plotted with the mean absorbance for each standard on the y-axis against the concentration on the x-axis and a best fit curve was drawn through the points on the graph by regression analysis. Unknown sample concentration was then back calculated using the standard curve.

1.2.7.3. *Estimation of caspase-3 in sciatic nerve and kidney*

Caspase-3 is also called as CPP-32 is an intracellular proenzyme belonging to the category of cysteine proteases. Cascade of events associated with apoptosis lead to the activation of caspase-3. The Caspase-3/CPP32 colorimetric assay kit involves the cleavage of cellular molecules that contain amino acid sequence DEVD. The assay involves detection of the chromophore \( p \)-nitroanilide (\( p \)NA) spectrophotometrically after its separation from the labelled DEVD-\( p \)NA substrate. The \( p \)NA colour intensity can be quantified using a micro plate reader at 405 nm. Activity of the caspase enzyme is directly proportional to the colour reaction.

**Assay procedure**

Sufficient amount DTT was mixed with the 2X reaction buffer immediately just before its usage (10 mM final concentration: add 10 \( \mu \)l of fresh 1.0 M DTT stock per 1 ml of 2X Reaction Buffer). Protect DEVD-\( p \)NA from light.

1. Cell pellet from the cells that undergone apoptosis was lysed with lysis buffer (25 \( \mu \)l lysis buffer is added to 1 \( \times \) \( 10^6 \) cells). After incubation for 10 minutes on ice, test samples were centrifuged at 10000 \( \times \) g for 1 minute to get clear supernatant with approximate protein concentration of 2-4 mg/ml.
2. 50 \( \mu \)l diluted cell lysate (containing 100-200 \( \mu \)g of total protein) is added to each well.
3. 50 \( \mu \)l of 2X reaction buffer (containing 10 mM DTT) was added to each sample.
4. 5 μl of the caspase-3 colorimetric substrate (4 mM DEVD-pNA) is added to each well and incubated at 37 °C for 1-2 hour.

5. Samples were analysed at 405 nm in a micro plate reader (BioRad, Xmerk). Caspase-3 activity was determined by comparing the results with the level of the non induced control.

1.2.8. Statistical analysis

Statistical analysis was performed by using GraphPad Prism (San Diego, CA). All the values were expressed as mean ± S.E.M. The data were analyzed using one way analysis of variance (ANOVA) (for biochemical analysis) followed by Tukey’s and two way ANOVA (mechanical allodynia, mechanical hyperalgesia, heat hyperalgesia) followed by Bonferroni’s tests. In all the tests, criterion for statistical significance was considered to be \( p<0.05 \).

1.3. Results

1.3.1. Effect of telmisartan, azilsartan and ramipril on body weight of STZ induced diabetic rats

Vehicle treated group did not show any significant effect on body weight as compared to naive group of animals throughout the study period. Single intraperitoneal injection of STZ (55 mg/kg) significantly \( (p<0.001) \) caused fall in body weight as compared to vehicle control group. Meanwhile, treatment with telmisartan (10 mg/kg), azilsartan (2 & 4 mg/kg) and ramipril (0.2 & 2.3 mg/kg) for 8 weeks significantly \( (p<0.05) \) restored the decrease in body weight as compared to diabetic control (STZ) animals (Fig. 1.2). However, telmisartan (5 mg/kg) failed to show significant effect on loss of body weight as compared to diabetic control animals. Further, per se treatment of higher dose of telmisartan (10 mg/kg), azilsartan (4 mg/kg) and ramipril (2.3 mg/kg) did not demonstrate any significant effect on body weight as compared to vehicle treated animals (data not shown).
Figure 1.2 Effect of telmisartan, azilsartan and ramipril on body weight of diabetic rats. Data presented as mean ± S.E.M. \(^a\) p<0.05 as compared to vehicle control, \(^b\) p<0.05 as compared to STZ control, \(^c\) p<0.05 as compared to T (5), \(^d\) p<0.05 as compared to A (2), (One-way ANOVA followed by Tukey's Multiple Comparison Test). STZ: streptozotocine; T: telmisartan; A: azilsartan; R: ramipril.

1.3.2. Effect of telmisartan, azilsartan and ramipril on food and water intake of STZ induced diabetic rats

Vehicle treatment did not display any significant alteration in food and water intake as compared to naive animals. Intraperitoneal STZ (55 mg/kg) administration significantly caused an increase in food intake (polyphagia), water intake (polydypsia) on day 56 (8\(^{th}\) week) as compared to vehicle treated group. However, 8 weeks treatment with telmisartan (5 & 10 mg/kg), azilsartan (2 & 4 mg/kg) and ramipril (0.2 & 2.3 mg/kg) did not demonstrate any significant (P<0.05) effect on food and water intake as compared to control (diabetic rats) (Fig. 1.3). Besides, telmisartan (10 mg/kg), azilsartan (4 mg/kg) and ramipril (2.3 mg/kg) per se treatment did not show any significant effect on food and water intake as compared to vehicle treated animals (data not shown).
Figure 1.3 Effect of telmisartan, azilsartan and ramipril on food and water intake of diabetic rats. Data expressed as mean ± S.E.M. \(^a p<0.05\) as compared to vehicle control. (One-way ANOVA followed by Tukey's Multiple Comparison Test). STZ: streptozotocine; T: telmisartan; A: azilsartan; R: ramipril.

1.3.3. Effect of telmisartan, azilsartan and ramipril on mechanical allodynia in STZ induced diabetic rats

No significant difference in mechanical allodynic response (paw withdrawal pressure) was observed between vehicle treated & naive group animals. Single STZ (55 mg/kg, i.p) administration significantly \((p<0.001)\) caused mechanical allodynia (decreased paw withdrawal threshold) on 4\(^{th}\), 6\(^{th}\) and 8\(^{th}\) week as compared to vehicle control group. However, 8 week treatment with telmisartan (5 & 10 mg/kg), azilsartan (2 & 4 mg/kg) and ramipril (0.2 & 2.3 mg/kg) significantly \((p<0.05)\) raised paw withdrawal threshold (an increased paw withdrawal pressure) on 6\(^{th}\) & 8\(^{th}\) weeks as compared to diabetic control group (Fig. 1.4). Further, telmisartan (10 mg/kg), azilsartan (4 mg/kg) and ramipril (2.3 mg/kg) per se treatment did not demonstrate any significant effect on mechanical alldonyia as compared to vehicle control group (data not shown).
Figure 1.4 Effect of telmisartan, azilsartan and ramipril on mechanical allodynia of diabetic rats in vonFrey test. Data expressed as mean ± S.E.M. 

\( ^{a} p<0.05 \) as compared to vehicle control,  
\( ^{b} p<0.05 \) as compared to STZ control,  
\( ^{c} p<0.05 \) as compared to T (5),  
\( ^{d} p<0.05 \) as compared to A (2),  
\( ^{e} p<0.05 \) as compared to R (0.2) (Two-way ANOVA followed by Bonferroni’s test). STZ: streptozotocine; T: telmisartan; A: azilsartan; R: ramipril.

### 1.3.4. Effect of telmisartan, azilsartan and ramipril on mechanical hyperalgesia of diabetic rats

No significant \( (p<0.05) \) difference in mechanical hyperalgesic response (paw withdrawal pressure) was observed between vehicle treated & naive group animals. Single i.p. injection of STZ (55 mg/kg) caused significant \( (p<0.001) \) mechanical hyperalgesia [decreased paw withdrawal threshold (pressure at which paw was withdrawn)] as compared to vehicle control group on 4\(^{th}\), 6\(^{th}\), and 8\(^{th}\) weeks. Treatment with telmisartan (10 mg/kg), azilsartan (4 mg/kg) and ramipril (2.3 mg/kg) significantly \( (p<0.001) \) attenuated mechanical hyperalgesia [raised paw withdrawal threshold (pressure at which paw was withdrawn)] as compared to STZ control on 4\(^{th}\), 6\(^{th}\) and 8\(^{th}\) weeks (Fig. 1.5). Besides, telmisartan (5 mg/kg), azilsartan (2 mg/kg) treatment significantly attenuated mechanical hyperalgesia from 6\(^{th}\) week onwards. Similarly, ramipril (0.2 mg/kg) treatment significantly reduced mechanical hyperalgesia on 8\(^{th}\) week as compared to STZ control. Further, telmisartan (10 mg/kg), azilsartan (4 mg/kg) and ramipril (2.3...
mg/kg) per se treatment did not show any significant effect on mechanical hyperalgesia as compared to vehicle control group (data not shown).

![Mechanical hyperalgesia](image)

**Figure 1.5 Effect of telmisartan, azilsartan and ramipril on mechanical hyperalgesia of diabetic rats in Randall-Selitto test.** Data expressed as mean ± S.E.M. 

- **a** $p<0.05$ as compared to vehicle control,
- **b** $p<0.05$ as compared to STZ control,
- **c** $p<0.05$ as compared to T (5),
- **d** $p<0.05$ as compared to R (0.2) (Two-way ANOVA followed by Bonferroni’s test).

STZ: streptozotocine; T: telmisartan; A: azilsartan; R: ramipril.

### 1.3.5. Effect of telmisartan, azilsartan and ramipril on thermal hyperalgesia of diabetic rats

There was no significance difference in thermal hyperalgesic response was observed between vehicle treated and naive group animals in hot plate test. Single i.p injection of STZ (55 mg/kg) resulted in significant ($p<0.001$) thermal hyperalgesia [as evidenced by decrease in paw withdrawal latency]. 8 weeks treatment with telmisartan (5 & 10 mg/kg), azilsartan (2 & 4 mg/kg) and ramipril (0.2 & 2.3 mg/kg) significantly ($p<0.05$) improved paw withdrawal latency as compared to STZ control on 6th and 8th weeks (Fig. 1.6). However, telmisartan (10 mg/kg), azilsartan (4 mg/kg) and ramipril (2.3 mg/kg) per se treatment did not show any significant effect in thermal hyperalgesia as compared to vehicle control group (data not shown).
Figure 1.6 Effect of telmisartan, azilsartan and ramipril on thermal hyperalgesia of diabetic rats in hot plate test. Data expressed as mean ± S.E.M. \( ^{2}p<0.05 \) as compared to vehicle control, \( ^{b}p<0.05 \) as compared to STZ control, \( ^{c}p<0.05 \) as compared to A (2), \( ^{d}p<0.05 \) as compared to R (0.2) (Two-way ANOVA followed by Bonferroni’s test). STZ: streptozotocine; T: telmisartan; A: azilsartan; R: ramipril.

1.3.6. Effect of telmisartan, azilsartan and ramipril on blood (glucose, creatinine, blood urea nitrogen, total proteins & serum albumin) and urine parameters (glomerular filtration rate) of STZ induced diabetic rats

Vehicle treatment did not demonstrate any significant \( (p<0.05) \) effect on any of the blood and urine parameters as compared to naive group animals. Single intraperitoneal injection of STZ (55 mg/kg) significantly \( (p<0.001) \) increased serum glucose, serum creatinine, blood urea nitrogen, and decreased serum total proteins, serum albumin, and glomerular filtration rate at the end of 8 weeks as compared to vehicle treatment (Table 1.2). Further, telmisartan (5 and 10 mg/kg), azilsartan (2 and 4 mg/kg) and ramipril (0.2 and 2.3 mg/kg) treatments did not demonstrate any significant \( (p<0.05) \) effect on serum glucose parameter as compared to STZ control group. Nevertheless, significant \( (p<0.05) \) reduction of serum creatinine, blood urea nitrogen as well as \( (p<0.05) \) increased serum total protein, serum albumin and glomerular filtration rate were observed with telmisartan (5 and 10 mg/kg), azilsartan (2 and 4 mg/kg) and ramipril (0.2 and 2.3 mg/kg) treatment as compared to STZ control. However, telmisartan (10 mg/kg), azilsartan (4 mg/kg) and ramipril (2.3 mg/kg) per se treatment did not show any significant effect on any of these blood and urine parameters as compared to vehicle control group (data not shown).
Table 1.2 Effect of telmisartan, azilsartan and ramipril on blood and urine parameters in diabetic rats

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Serum Glucose (mg/dl) (% Vehicle control)</th>
<th>Serum creatinine (mg/dl) (% Vehicle control)</th>
<th>Blood urea nitrogen (mg/dl) (% Vehicle control)</th>
<th>Serum total proteins (g/dl) (% Vehicle control)</th>
<th>Serum albumin (g/dl) (% Vehicle control)</th>
<th>Glomerular filtration rate (% Vehicle control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>100.12 ± 4.10 (103.16)</td>
<td>0.62 ± 0.03 (95.13)</td>
<td>21.53 ± 0.56 (98.37)</td>
<td>7.35 ± 0.14 (98.39)</td>
<td>5.55 ± 0.12 (97.14)</td>
<td>1.06 ± 0.09 (98.39)</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>99.76 ± 2.90 (100)</td>
<td>0.65 ± 0.03 (100)</td>
<td>21.89 ± 0.33 (100)</td>
<td>7.45 ± 0.16 (100)</td>
<td>5.70 ± 0.06 (100)</td>
<td>1.09 ± 0.11 (100)</td>
</tr>
<tr>
<td>STZ Control</td>
<td>457.31 ± 11.40 (458.38)</td>
<td>1.41 ± 0.04 (216.81)</td>
<td>50.22 ± 1.21 (40.51)</td>
<td>4.68 ± 0.12 (244.84)</td>
<td>3.39 ± 0.10 (34.68)</td>
<td>0.43 ± 0.02 (244.84)</td>
</tr>
<tr>
<td>STZ+T(5)</td>
<td>431.29 ± 16.80 (432.39)</td>
<td>1.02 ± 0.05 (156.80)</td>
<td>43.14 ± 0.77 (49.31)</td>
<td>5.94 ± 0.19 (206.45)</td>
<td>4.55 ± 0.13 (79.95)</td>
<td>0.73 ± 0.03 (206.45)</td>
</tr>
<tr>
<td>STZ+T(10)</td>
<td>396.08 ± 7.10 (397.01)</td>
<td>0.78 ± 0.03 (119.40)</td>
<td>38.62 ± 1.24 (60.97)</td>
<td>6.35 ± 0.18 (185.16)</td>
<td>4.93 ± 0.15 (66.31)</td>
<td>0.77 ± 0.02 (185.16)</td>
</tr>
<tr>
<td>STZ+A(2)</td>
<td>427.78 ± 16.60 (424.76)</td>
<td>1.07 ± 0.10 (164.69)</td>
<td>41.95 ± 0.89 (185.16)</td>
<td>5.79 ± 0.15 (210)</td>
<td>4.56 ± 0.15 (80.28)</td>
<td>0.69 ± 0.02 (210)</td>
</tr>
<tr>
<td>STZ+A(4)</td>
<td>402.42 ± 19.20 (403.36)</td>
<td>0.87 ± 0.02 (134.10)</td>
<td>35.46 ± 0.86 (180.65)</td>
<td>6.76 ± 0.07 (180.65)</td>
<td>4.97 ± 0.12 (87.00)</td>
<td>0.74 ± 0.02 (180.65)</td>
</tr>
<tr>
<td>STZ+R(0.2)</td>
<td>441.66 ± 14.50 (442.69)</td>
<td>0.90 ± 0.02 (138.19)</td>
<td>45.92 ± 1.17 (72.18)</td>
<td>5.30 ± 0.15 (158.39)</td>
<td>4.68 ± 0.09 (82.03)</td>
<td>0.54 ± 0.02 (158.39)</td>
</tr>
<tr>
<td>STZ+R(2.3)</td>
<td>425.20 ± 19.50 (426.19)</td>
<td>0.85 ± 0.02 (131.03)</td>
<td>41.02 ± 1.00 (103.73)</td>
<td>5.67 ± 0.11 (102.59)</td>
<td>4.98 ± 0.11 (87.15)</td>
<td>0.61 ± 0.03 (102.59)</td>
</tr>
</tbody>
</table>

Data expressed as mean ± S.E.M. *p<0.05 as compared to vehicle control, †p<0.05 as compared to STZ control, ‡p<0.05 as compared to T (5), §p<0.05 as compared to A (2), ‡p<0.05 as compared to R (0.2) (One-way ANOVA followed by Tukey's Multiple Comparison Test). STZ: streptozotocine; T: telmisartan; A: azilsartan; R: ramipril.
1.3.7. Effect of telmisartan, azilsartan and ramipril on motor nerve conduction velocity (MNCV) on sciatic nerves of STZ induced diabetic rats

No significant difference in MNCV of sciatic nerve was observed between vehicle treated and naive group animals. Single intraperitoneal injection of streptozotocine (55 mg/kg) significantly decreased MNCV at the end of 8 weeks as compared to vehicle control group (Fig 1.7). Further, unlike lower dose, higher dose of telmisartan (10 mg/kg), azilsartan (4 mg/kg) and ramipril (2.3 mg/kg) treatments for 8 weeks significantly \( p<0.05 \) improved nerve conduction velocity as compared to STZ control rats. However, telmisartan (10 mg/kg), azilsartan (4 mg/kg) and ramipril (2.3 mg/kg) per se treatment did not show any significant effect in nerve conduction velocity as compared to vehicle control group (data not shown).

![Graph showing MNCV]

**Figure 1.7** Effect of telmisartan, azilsartan and ramipril on MNCV of diabetic rats. Data expressed as mean ± S.E.M. \(^{a}\) \( p<0.05 \) as compared to vehicle control, \(^{b}\) \( p<0.05 \) as compared to STZ control (One-way ANOVA followed by Tukey’s Multiple Comparison Test). STZ: streptozotocine; T: telmisartan; A: azilsartan, R: ramipril.
1.3.8. Effect of telmisartan, azilsartan and ramipril on sciatic nerve and kidney oxidative damage (LPO, GSH, SOD and catalase levels) in diabetic rats

No significant difference in oxidative stress parameters (LPO, GSH, SOD & catalase) in both sciatic nerve and kidney was observed between vehicle treated and naive group animals. Single i.p STZ (55 mg/kg) injection significantly (p<0.001) increased lipid peroxidation, reduced GSH, SOD & catalase in sciatic nerve as well as kidney as compared to the vehicle control group. However, 8 week treatment with telmisartan (10 mg/kg), azilsartan (4 mg/kg) and ramipril (2.3 mg/kg) significantly (p<0.05) attenuated oxidative stress parameters and caused antioxidant like effects in both sciatic nerve and kidneys as evidenced by decrease in LPO, restoration of GSH, SOD and catalase activities as compared to the STZ control rats (Table 1.3). Further, treatment with telmisartan (5 mg/kg) significantly attenuated LPO but failed to reverse GSH, SOD and catalase enzymes activities in both sciatic nerve and kidneys as compared to STZ control rats. Azilsartan (2 mg/kg) treatment significantly (p<0.05) decreased LPO and restored GSH, SOD and catalase activities as compared to the STZ control rats. However, ramipril (0.2 mg/kg) treatment did not demonstrate any significant effect on any of the oxidative stress parameters in both sciatic nerve and kidneys as compared to diabetic rats. However, telmisartan (10 mg/kg), azilsartan (4 mg/kg) and ramipril (2.3 mg/kg) per se treatment did not show any significant effect in oxidative stress parameters in sciatic nerve and kidney as compared to vehicle control group (data not shown).
<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>MDA (nmol MDA/mg pr) (% Vehicle control)</th>
<th>Reduced glutathione (nmol of GSH/mg pr) (% Vehicle control)</th>
<th>Superoxide dismutase (U/min/mg Pr) (% Vehicle control)</th>
<th>Catalase (μmol of H₂O₂ decomposed/min/mg pr) (% Vehicle control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sciatic nerve</td>
<td>Kidney</td>
<td>Sciatic nerve</td>
<td>Kidney</td>
</tr>
<tr>
<td>Naive</td>
<td>1.83 ± 0.14</td>
<td>0.468 ± 0.05 (98.39)</td>
<td>31.22 ± 1.07 (102.46)</td>
<td>22.24 ± 0.81 (102.46)</td>
</tr>
<tr>
<td>Vehicle Control</td>
<td>1.74 ± 0.16</td>
<td>0.488 ± 0.06 (100)</td>
<td>32.11 ± 2.08 (100)</td>
<td>20.05 ± 0.88 (100)</td>
</tr>
<tr>
<td>STZ Control</td>
<td>3.99 ± 0.13 *</td>
<td>0.895 ± 0.07 * (244.84)</td>
<td>9.68 ± 0.93 * (40.51)</td>
<td>7.80 ± 0.49 * (40.51)</td>
</tr>
<tr>
<td>STZ+T(5)</td>
<td>3.28 ± 0.15 b</td>
<td>0.717 ± 0.03 (206.45)</td>
<td>12.45 ± 0.93 (49.31)</td>
<td>9.65 ± 0.51 (49.31)</td>
</tr>
<tr>
<td>STZ+T(10)</td>
<td>2.56 ± 0.11 b,c</td>
<td>0.520 ± 0.02 b,c (185.16)</td>
<td>18.50 ± 1.83 b,c (60.97)</td>
<td>13.82 ± 0.15 b,c (60.97)</td>
</tr>
<tr>
<td>STZ+A(2)</td>
<td>3.18 ± 0.13 b</td>
<td>0.694 ± 0.04 (210)</td>
<td>12.25 ± 0.82 (50.92)</td>
<td>10.55 ± 1.26 b (50.92)</td>
</tr>
<tr>
<td>STZ+A(4)</td>
<td>2.43 ± 0.20 b,d</td>
<td>0.494 ± 0.02 b,d (180.65)</td>
<td>18.26 ± 1.3 b,d (60.55)</td>
<td>14.34 ± 0.86 b,d (60.55)</td>
</tr>
<tr>
<td>STZ+R(0.2)</td>
<td>3.47 ± 0.13 a</td>
<td>0.726 ± 0.02 (158.39)</td>
<td>9.91 ± 0.83 (72.18)</td>
<td>8.48 ± 0.24 (72.18)</td>
</tr>
<tr>
<td>STZ+R(2.3)</td>
<td>3.17 ± 0.18 b</td>
<td>0.608 ± 0.03 b (102.58)</td>
<td>13.22 ± 0.86 b (103.73)</td>
<td>10.57 ± 0.15 b (103.73)</td>
</tr>
</tbody>
</table>

Data expressed as mean ± S.E.M. *p<0.05 as compared to vehicle control, †p<0.05 as compared to STZ control, ‡p<0.05 as compared to T (5), §p<0.05 as compared to A (2) (One-way ANOVA followed by Tukey’s Multiple Comparison Test). STZ: streptozotocin; T: telmisartan; A: azilsartan; R: ramipril.
1.3.9. Effect of telmisartan, azilsartan and ramipril on TNF-α levels in sciatic nerve and kidney of diabetic rats

No significant difference was observed in TNF-α level in both sciatic nerve and kidney between vehicle treated and naive group animals. Vehicle treatment did not show any significant ($p<0.05$) effect on TNF-α level in both sciatic nerve and kidney as compared to naive group. Single i.p STZ (55 mg/kg) injection significantly ($p<0.001$) raised TNF-α level in sciatic nerve as well as in kidney as compared to the vehicle treated group. However, 8 week treatment with telmisartan (5 & 10 mg/kg), azilsartan (2 & 4 mg/kg) and ramipril (0.2 & 2.3 mg/kg) significantly ($p<0.05$) attenuated TNF-α levels and caused antiinflammatory like effects in both sciatic nerve and kidneys as compared to the STZ control rats (Fig 1.8).

**Figure 1.8** Effect of telmisartan, azilsartan and ramipril on TNF-α level of diabetic rats. Data expressed as mean ± S.E.M. $^a p<0.05$ as compared to vehicle control, $^b p<0.05$ as compared to STZ control, $^c p<0.05$ as compared to A (2), $^d p<0.05$ as compared to R (0.2) (One-way ANOVA followed by Tukey’s Multiple Comparison Test). STZ: streptozotocine; T: telmisartan; A: azilsartan, R: ramipril.
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1.3.10. Effect of telmisartan, azilsartan and ramipril on caspase-3 activity in sciatic nerve and kidney of diabetic rats

Vehicle treatment did not demonstrate any significant \( p<0.05 \) effect on caspase-3 activity in both sciatic nerve and kidney as compared to naive group. Single i.p. STZ (55 mg/kg) injection significantly increased caspase-3 activity in sciatic nerve as well as in kidney as compared to the vehicle control group. However, 8 week treatment with telmisartan (5 & 10 mg/kg), azilsartan (2 & 4 mg/kg) and ramipril (2.3 mg/kg) significantly \( p<0.05 \) attenuated caspase-3 activity and caused antiapoptotic like effects in both sciatic nerve and kidneys as compared to the STZ control rats (Fig. 1.9).

![Graph showing caspase-3 activity in sciatic nerve and kidney](image)

Figure 1.9 Effect of telmisartan, azilsartan and ramipril on caspase-3 activity of diabetic rats. Data expressed as mean ± S.E.M. \(^a\)\( p<0.05 \) as compared to vehicle control, \(^b\)\( p<0.05 \) as compared to STZ control (One-way ANOVA followed by Tukey's Multiple Comparison Test). STZ: streptozotocine; T: telmisartan; A: azilsartan, R: ramipril.

1.3.11. Effect of telmisartan, azilsartan and ramipril on H&S stain of dorsal root ganglia and kidney in diabetic rats.

Vehicle treatment did not demonstrate any significant effect on histological alterations as compared to naive group in dorsal root ganglia (both A and B type cells) and kidney sections (intact glomeruli with normal mesangial matrix) (Fig. 1.10 A, B & 1.11A, B). Single i.p STZ (55 mg/kg) injection resulted in large vacuolated cells in DRG (Fig. 1.10C) and prominent thickening of glomerular...
membrane, mesangial matrix expansion as compared to vehicle treated group (Fig. 1.11C). However, treatment with telmisartan (5 & 10 mg/kg), azilsartan (2 & 4 mg/kg) and ramipril (0.2 & 2.3 mg/kg) treatment for 8 weeks significantly attenuated these pathological alterations as compared to diabetic rats (Fig. 1.10 D-I) displaying improved DRG neuronal & kidney glomerular structures.

Figure 1.10 Effect of telmisartan, azilsartan and ramipril on DRG histopathological analysis of STZ treated animals (H&E staining). Photomicrographs of DRG sections (magnification 40x) of rat. There are two types of cell present in DRG neurons: A type cells with large intensely stained central nucleus with granular cytoplasm (red arrow in C), where as B type cells contain peripherally located nuclei, neurons tend to be small and with large vacuole (yellow arrow in C). (A) naive & (B) vehicle control: normal A and B type cells; whereas in (C) Diabetic control (STZ 55 mg/kg), (D) STZ+ T (5), (E) STZ+ T (5), (F) STZ+ A (2), (G) STZ+ A (4), (H) STZ+ R (0.2) and (I) STZ+ R (2.3) groups small neurons with large vacuoles are present.
Figure 1.11 Effect of telmisartan, azilsartan and ramipril on kidney histopathological analysis of STZ treated animals (H&E staining). Photomicrographs of glomeruli sections (magnification 40x) of rat. (A) naïve: glomeruli are intact; (B) vehicle control: glomeruli are intact; (C) Diabetic control (STZ 55 mg/kg): glomeruli basement thickened and mesangial matrix expanded; (D) STZ+ T (5): moderately thickened basement membrane; (E) STZ+ T (5): mild thickened basement membrane and less mesangial matrix expansion; (F) STZ+ A (2): moderately thickened basement membrane; (G) STZ+ A (4): mild thickened basement membrane and less mesangial matrix expansion; (H) STZ+ R (0.2): moderately thickened basement membrane; (I) STZ+ R (2.3): mild thickened basement membrane and less mesangial matrix expansion;
1.4. Discussion

Diabetic microvascular complications like neuropathy and nephropathy involve complex pathophysiological changes in metabolic, vascular, immunological and neurohormonal pathways (Brownlee 2001; Cameron et al. 2001). Moderate increase in blood glucose levels is sufficient to activate renin angiotensin aldosterone system as seen in early diabetes mellitus (Hayashi et al. 2010). Increased levels of angiotensin-II are directly responsible for an increased ROS production leading to oxidative stress, inflammation and other neural and vascular related dysfunctions (Davidson et al. 2012).

In the present study, intraperitoneal injection of STZ elevated blood glucose, decreased body weight, increased food and water intake suggesting diabetic like complications. Besides, decreased paw withdrawal threshold for mechanical stimuli as in Randall-Selitto test (mechanical hyperalgesia), vonfrey hair test (mechanical allodynia) and shortened paw withdrawal latency in hot plate test (heat hyperalgesia) have been observed in the present study indicates neuropathy like symptoms. Further, increased serum creatinine, blood urea nitrogen and reduced glomerular filtration rat, serum albumin indicates the development of nephropathy. These results are in well agreement with the previous studies indicating the development of diabetic complications with single i.p injection of streptozotocine (Kumar et al. 2007; Kumar and Addepalli 2011). In the present study, treatment with telmisartan, azilsartan and ramipril caused attenuation of decrease in paw withdrawal threshold and improvement in paw withdrawal latency suggesting their protective effect. However, blood glucose, body weight, food and water intake did not change significantly in telmisartan, azilsartan and ramipril treated groups as compared to diabetic control animals. This could be due to their (telmisartan, azilsartan and ramipril) direct protective actions instead of affecting blood glucose levels.

Earlier, clinical studies also revealed the role of RAS in pain processing and provided equivocal results (Kalra et al. 2008). Direct microinjection of angiotensin-II in to the supra-spinal regions (caudal ventrolateral medulla) of male wistar rats resulted in hyperalgesia which was ameliorated with local losartan administration (Marques-Lopes et al. 2009). Earlier, presence of AT1
receptors have been shown in superficial dorsal horn lamina I and II, dorsal root ganglia, sciatic nerves (Pavel et al. 2008) and injury to sciatic nerve resulted in accumulation of AT1 receptors near the damaged area implying the anterograde transport of these receptors. Further, lamina I and II are the major areas where C and Aδ nociceptive fibers terminate. This further supports the hypothesis of an involvement of angiotensin-II in nociception. Besides, continuous sub-pressor dose of angiotensin-II is sufficient to induce injury of primary sensory neurons via reduction in blood flow to DRG of these neurons. This reduction in blood flow probably disturbs the metabolic needs and activity of neurons resulting in neuronal damage (Pavel et al. 2013) and pain.

Besides, single intraperitoneal injection of STZ increases blood urea nitrogen (BUN), serum creatinine, reduced the serum protein, serum albumin and GFR which are characteristics of diabetic nephropathy. Further, treatment with telmisartan, azilsartan and ramipril significantly reversed serum creatinine, BUN, serum protein and GFR levels indicating their direct renoprotection action independent of their effects on blood glucose. In diabetes, initially it is well known that because of glomerular hyper perfusion, an increased GFR has been observed that causing microalbuminuria which if unchecked will lead to macroalbuminuria subsequently decrease in GFR owing to reduced kidney functions (Meeme and Kasozi 2009). Glomerular mesangial hypertrophy has also been observed in diabetic nephropathy. Further, activated RAS also leads to increased macrophage recruitment via monocyte chemo attractant protein-1 (MCP-1) in epithelial cells of diabetic kidney. Besides, Ang-II also directly activates different other immune, inflammatory mediators (Ruiz-Ortega et al. 2001), as well as increase ROS production and causes autophagy by increasing expression of LC3-2 & beclin-1 autophagic genes in podocytes (Yadav et al. 2010).

These results strongly support the involvement of RAS in diabetic neuropathy and nephropathy. Besides, these results are in line with the previous studies demonstrating the efficacy of ACE-I and ARBs in improving the neural and kidney dysfunction (Jaggi and Singh 2011; Baltatzi et al. 2011; Khan et al. 2009; Coppey et al. 2006).
Evidence has shown that improvement of vascular, nerve function and nerve blood flow in streptozotocin induced diabetic rats with ARBs even after establishment of diabetes (Coppey et al. 2006). Diabetic neuropathy results in decreased nerve conduction velocity because of, reduced nerve blood flow secondary to vasoconstriction, reduced energy production because of decrease in Na/K-ATPase activity, increase in free radical production. In the present study, approximately 50% reduction in nerve conduction velocity has been observed in diabetic animals. Treatment with telmisartan, azilsartan and ramipril significantly improved these results in motor nerve conduction velocity studies and improved the nerve function suggesting their therapeutic importance.

Antidiabetic effects of ARBs have been attributed partly to their improvement in blood flow to skeletal muscle. Evidence also shown that tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) is crucial step in insulin signaling. Angiotensin-II stimulates the serine phosphorylation of IRS-1 thereby prevents tyrosine phosphorylation of IRS-1 and interferes with the insulin signaling (Hayashi et al. 2014). Besides, telmisartan in particular is known to activate PPAR gamma mechanism and have shown some beneficial effects in diabetes (Kurtz and Pravenec 2004). Further, there are studies reporting an inhibition of NAD(P)H oxidase mediated production of ROS leading to decrease in oxidative stress (Cao et al. 2009; Nlandu Khodo et al. 2012; Sedeek et al. 2013). Besides, insulin sensitizing effects of azilsartan have been reported in diabetic rats and dogs (Kusumoto et al. 2011; Zhao et al. 2011; Lastra et al. 2013) and pharmacological profile of azilsartan is very much promising in recent clinical trials (White et al. 2011). Even though the preliminary studies of azilsartan are promising the efficacy of azilsartan on PPAR gamma needs to be established fully (Iwai et al. 2007; Zhao et al. 2011).

Various classical metabolic pathways (increased polyol pathway activity, protein kinase c, advanced glycation end product formation activity) ultimately converge to cause oxidative stress leading to diabetic complications (neuropathy, nephropathy and retinopathy) (Brownlee 2005). There is also an increase in ROS generation through the activation of NAD(P)H oxidase by angiotensin-II will also add to an increased oxidative stress in diabetic complications. And various ARBs have been shown to decrease angiotensin induced increase in ROS generation.
implying the antioxidant potentials of these classes of drugs. Efficacy of ACE inhibitors and ARBs via inhibition of NAD(P)H oxidase has shown in diabetic complications. Further, NAD(P)H oxidase and ROS are well known to be associated in the maintenance of NP after nerve damage (Kallenborn-Gerhardt et al. 2012) and amelioration of diabetic neuropathy with the agents which can inhibit oxidative stress through the NAD(P)H oxidase mechanism were observed in rats. Not only neurons but kidney also expresses the abundant amount of NAD(P)H oxidase and plays crucial role in diabetic nephropathy (Asaba et al. 2005). So, inhibiting the oxidative stress induced by this enzyme system will ultimately protect the neurons and kidneys from excess glucose induced tissue damage via ROS. Excessive glucose levels increase the metabolic flux via mitochondrial electron transport chain leading to an increased release of superoxide radical. These superoxide radicals have been known to enhance the production of other free radicals like H$_2$O$_2$, OH$^-$ radicals and peroxide radical (by combing with nitric oxide) acts directly on DNA and causes cell death. In our present study, telmisartan, azilsartan and ramipril have also been shown to decrease the lipid peroxidation and restored antioxidant defense enzymes like GSH, SOD and catalase in both sciatic nerve and kidney. This postulates the antioxidant potential of these drugs in diabetic complications.

Growing body of evidence shifts the focus of the diabetic complications from classical metabolic abnormalities to inflammatory conditions (King 2008; Navarro-Gonzalez and Mora-Fernandez 2008). Thus, angiotensin-II which has proinflammatory properties has got tremendous importance in the disease progression in diabetic patients. Increased flux through polyol pathway activates PKC and reduces the function Na/K-ATPase , activation of MAPKinases, NF-kB, TGF-β in endothelial cells initiates inflammatory responses.

Angiotensin-II has been shown to regulate the cellular growth and differentiation, induces inflammation directly or via different immune pathways (Suzuki et al. 2003; Benigni et al. 2010). Treatments with ARBs have been shown to decrease the inflammatory markers (Silveira et al. 2013; Cote et al. 2014; Matsumoto et al. 2014). Further, cross talk exists between oxidative stress and inflammation. Increased oxidative stress activates glial cells and there by release the different inflammatory mediators (Ogundele et al. 2014). But, the effect of ACE-I on this
inflammatory response is a matter of debate. In the present study, telmisartan, azilsartan have been shown to decrease the inflammatory makers in sciatic nerve and kidney homogenate. Existence of AT1 receptors in inflammatory and immune (glial) cells has been reported (Nataraj et al. 1999; Fuchtbauer et al. 2011; Zhang and Crowley 2013). Further, efficacy of ARBs in nerve injury induced increase in inflammatory markers have been shown in chronic constriction injury models implying the potential role of ARBs in the neuroinflammatory conditions (Jaggi and Singh 2011). Further, researchers have shown that telmisartan and azilsartan possess efficacy towards PPARγ receptors and exhibits antiinflammatory like actions.

Angiotensin-II has been shown to induce MAPK pathway leading to an activation of different immune and inflammatory cells. Spinally located AT1 receptors are known to induce pain via p38-MAPK mechanism which mainly linked to cellular stress and proinflammatory mechanisms. p38-MAPK induction also increases the expression of proinflammatory cytokines (IL-6 & TNF-α) which are inhibited by ARB treatment (Nemoto et al. 2013). In the current study, diabetic animals displayed an increase TNF-α level in both sciatic nerves and kidneys. Further, treatments with telmisartan, azilsartan and ramipril have been shown to ameliorate TNF-α level in sciatic nerve and kidneys as well indicating their antiinflammatory like actions.

Diabetes induced glomerular hypertrophy and mesangial cell expansions have been observed in kidney histology. Besides, DRG neurons undergo morphological changes leading to large vacuolated cytoplasm. Treatment with telmisartan, azilsartan and ramipril attenuated these changes showing their protective effects.

Increased mitochondrial dysfunction and altered ETC chain will lead to production of various free radicals and leakage of cytochrome-c into the cytoplasm. This will unleash a cascade of events for apoptosis via activation of different caspases which ultimately lead to caspase-3 mediated apoptosis. In this study, telmisartan, azilsartan and ramipril treatment significantly opposed the apoptosis as evidenced by the reduced caspase-3 activity in neural and kidney.
Evidence has shown the presence of AT1 receptors in peripheral, spinal and supra-spinal sites. So, at present it is not clear whether the effect is because of spinal, supra spinal RAS involvement in the effects of telmisartan, azilsartan and ramipril. Having known the lipophilicity, capacity to cross the BBB makes it to think that we cannot completely rule out the possibility of central RAS involvement in the observed protective actions of these drugs. However, additional studies are needed to confirm the precise mechanisms through which these drugs exert their neuroprotective actions in diabetes induced neuropathy and nephropathy.

Improvement of vascular and neural functions remains the area to be focused besides tight blood glucose regulation in order to prevent the progression of the diabetes. Further, ARBs/ACE-I inhibitors are well tolerated and first line agent for monotherapy and combination therapy in hypertensive patients. Treatment with agents that can control the blood glucose levels along with ARBs/ACE-I could be useful approach in combating the diabetes induced neuropathy and nephropathy (Fig 1.12).

Overall, azilsartan have been shown to possess better protective effect in STZ induced neuropathy and nephropathy. This could be attributed to its AT1 receptor binding profiles as the carboxyl moiety present in its structure aids in tight binding with these receptors there by exerts longer lasting effects. Further, additional studies are warranted to delineate the exact mechanisms involved in diabetes induced neuropathy and nephropathy.
Possible targets of action of telmisartan, azilsartan and ramipril

Figure 1.12 Possible targets of action of telmisartan, azilsartan and ramipril in STZ induced neuropathic pain and nephropathy in rats.