CHAPTER 3

Ameliorative potential of pioglitazone, ceftriaxone and their interaction in rat model of neuropathic pain: Targeting PPARγ and GLT-1 pathways

3.1. Introduction

Neuropathic pain (NP) is a rapidly growing challenging problem because of wider prevalence among diabetes as well as inadequate treatment options (Tan et al. 2010). Intricate interplay between wide variety of the pathophysiological mechanisms involved in NP makes it difficult to design effective therapeutic strategies (Baron et al. 2010). So, there is an imperative need for the development of better therapeutic regimen with an improved efficacy and minimal side effect profiles.

Peroxisome proliferator-activated receptor gamma (PPARγ) receptors and their downstream mechanisms are well involved in nociception (Maeda and Kishioka 2009). Inflammatory mediators released from activated macrophages, glial cells play a critical role in the pathology of both inflammatory and NP. PPARγ modulators have been well suggested to transrepress the expression of these inflammatory cascades via different cellular and molecular mechanisms (Freitag and Miller 2014; Park et al. 2007; Takahashi et al. 2011). Further, their protective effects in neuroinflammatory, oxidative stress process have been reported in different neuronal disorders (Culman et al. 2007; Kiaei et al. 2005; Jin et al. 2013). Pioglitazone, a PPARγ agonist with better blood brain barrier permeability, is known to affect neuro-immune mechanisms in the spinal cord (Griggs et al. 2015). Oxidative stress and free radicals alter mitochondrial enzyme complex activities, caspase activation (Bennett et al. 2014). Mitochondrial impairment that causes deficiency of energy production has also been suggested in diabetes induced neuropathy (Chowdhury et al. 2010; Fidanboylu et al. 2011). Further, mitochondrial poisons worsen by targeting the sensory neuronal mitochondria (Xiao and Bennett 2012). However, mitochondrial impairment in NP has remained unexplored and the data related to mitochondrial enzyme complexes are sparse. Traditionally, PPARγ is known for its antidiabetic, antiinflammatory activities, but studies suggest that activation of PPARγ is involved in the alteration of glutamate transport (Romera et al. 2007). Further, pioglitazone has
also been shown to be associated with non-genomic PPARγ mechanism (Griggs et al. 2015).

Glutamate concentration at synapse is maintained and totally controlled by glutamate/ excitatory amino acid transporters (EAAT). Of the five types (EAAT 1-5) of these transporters cloned till date, EAAT2 (GLT-1) is responsible for more than 90% of glutamate clearance in central nervous system (Osikowicz et al. 2013). Growing body of evidence reported a considerable decrease in the spinal cord expression of GLT-1 in different neurodegenerative conditions including NP (Amin et al. 2012; Maragakis and Rothstein 2006). Selectively increasing the expression of this transporter has been suggested as one of the protective mechanism in preventing glutamate mediated neuronal toxicity (Nicholson et al. 2014). NP has been known to be associated with an increased release of glutamate in synaptic cleft thereby causing hyperexcitability of second order neurons (Osikowicz et al. 2013). Indeed, glutamate antagonists have been tried for NP relief with limited success because of their narrow therapeutic window and associated side effects (Sang et al. 2002). So, combination therapy could be an alternative strategy for an effective pain relief. Therefore, the present study has been designed with a hypothesis that simultaneously targeting PPARγ and GLT-1 could be a useful treatment option for NP. Therefore, ceftriaxone has been selected for this study.

Ceftriaxone, a β-lactam antibiotic, specifically up-regulate GLT-1 expression and has been shown to be beneficial in different neuronal disorders (Amin et al. 2012; Ramos et al. 2010). Despite the fact that ceftriaxone is effective in different NP models data showing its efficacy in spinal nerve ligation (SNL) model is lacking and needs further investigation. Hence, the current study has been intended to investigate the protective effect of pioglitazone and its interaction with ceftriaxone in SNL induced NP.

3.2. Materials and Methods

3.2.1. Animals

Male SD rats (180–220 g) bred in Central Animal House, Panjab University, Chandigarh were used in this study. Animals were adapted to the testing conditions prior to start of the experiments. They were maintained on 12-h
light/dark cycle and have free access to food and water. Ethical clearance from IAEC of Panjab University was obtained prior to the start of the experiment (IAEC/504/UIPS-7/2/4/14) and carried out as per the guidelines of Indian National Science Academy guidelines for the use and care of experimental animals. All the behavioral studies were carried out by a person who is blinded to the treatment groups.

3.2.2. Spinal nerve ligation

Refer to chapter 2 (2.2.2)

3.2.3. Drug and treatment schedule

Study protocol included ten treatment groups, with ten animals in each group. Pioglitazone, ceftriaxone (Panacea Biotech, Mohali) were included in the present study. Pioglitazone was suspended in 0.25% w/v sodium carboxy methyl cellulose (CMC) and ceftriaxone was dissolved in normal saline. Animals were divided into different groups (Table 3.1) and received respective drug treatments daily (10:00 am), for 28 days beginning on the day after SNL. Drugs were administered by the intraperitoneal (i.p.) route as per body weight (5 ml/kg). Earlier literature reports formed the basis for drug dose selection (Amin et al. 2012; Jia et al. 2010). All the behavioral experiments were performed 30 min after the drug administration.
Table 3.1 Treatment groups

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Group</th>
<th>Treatment (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Naïve</td>
<td>Healthy animals (no treatment)</td>
</tr>
<tr>
<td>2</td>
<td>Sham</td>
<td>Surgery performed, vehicle administered</td>
</tr>
<tr>
<td>3</td>
<td>SNL (Control)</td>
<td>SNL + vehicle administered for 28 days</td>
</tr>
<tr>
<td>4</td>
<td>SNL + Pio (5)</td>
<td>SNL + Pioglitazone (5 mg/kg, i.p.)</td>
</tr>
<tr>
<td>5</td>
<td>SNL + Pio (10)</td>
<td>SNL + Pioglitazone (10 mg/kg, i.p.)</td>
</tr>
<tr>
<td>6</td>
<td>SNL + Pio (20)</td>
<td>SNL + Pioglitazone (20 mg/kg, i.p.)</td>
</tr>
<tr>
<td>7</td>
<td>SNL + Cef (100)</td>
<td>SNL + Ceftriaxone (100 mg/kg, i.p.)</td>
</tr>
<tr>
<td>8</td>
<td>SNL + Cef (200)</td>
<td>SNL + Ceftriaxone (200 mg/kg, i.p.)</td>
</tr>
<tr>
<td>9</td>
<td>SNL + Pio (5) + Cef (100)</td>
<td>SNL + Pioglitazone (5 mg/kg, i.p.) + Ceftriaxone (100 mg/kg, i.p.)</td>
</tr>
<tr>
<td>10</td>
<td>SNL + Pio (20) + Cef (200)</td>
<td>SNL + Pioglitazone (20 mg/kg, i.p.) + Ceftriaxone (200 mg/kg, i.p.)</td>
</tr>
</tbody>
</table>

3.2.4. Behavioural Assessments

3.2.4.1. Mechanical allodynia

Refer to chapter 1 (1.2.4.3)

3.2.4.2. Mechanical hyperalgesia

Refer to chapter 1 (1.2.4.4)

3.2.4.3. Cold allodynia

Refer to chapter 2 (2.2.4.3)

3.2.5. Biochemical assessments

3.2.5.1. Dissection and homogenization

Immediately after last behavioral assessment on day 28, cervical dislocation was employed to sacrifice all the animals. Spinal cord from L5-L6 region was rapidly dissected out, placed on ice and stored at -20° C. For the biochemical
estimations, 10% (w/v) tissue homogenates were prepared in 10 mM phosphate buffer (pH 7.4) using homogenizer (Remi motors, India) and centrifuged at 10,000 × g at 4°C for 15 min (Sigma, UK). Supernatant obtained was used for biochemical assays. Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, USA) was used for the estimation of biochemical and mitochondrial parameters.

3.2.5.2. Measurement of endogenous antioxidant profile

3.2.5.2.1. Measurement of lipid peroxidation
Refer to chapter 1 (1.2.7.1.1).

3.2.5.2.2. Estimation of nitrite
Refer to chapter 2 (2.2.5.2.2)

3.2.5.2.3. Superoxide dismutase activity
Refer to chapter 1 (1.2.7.1.3)

3.2.5.2.4. Glutathione assay
Refer to chapter 1 (1.2.7.1.2)

3.2.5.2.5. Protein estimation
Refer to chapter 1 (1.2.7.1.5)

3.2.5.3. Estimation of TNF-α level in spinal cord
Refer to chapter 1 (1.2.7.2)

3.2.5.4. Estimation of caspase-3 activity in spinal cord
Refer to chapter 1 (1.2.7.3)

3.2.6. Mitochondrial parameter assessment

3.2.6.1. Isolation of spinal cord mitochondria
Lumbar spinal cord mitochondria were obtained by the method as described by Berman and Hastings. Spinal cord of animals were homogenized in isolation buffer with EGTA (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, and pH 7.2). Homogenates were centrifuged at 13,000 × g for 5 min at 4°C. Isolation buffer containing EGTA was used to suspend the pellets & spun again at 13,000 × g for 5 min. The resulting supernatants were transferred and mixed with isolation buffer containing EGTA and centrifuged again at 13,000 × g for 10 min. Pure mitochondria present in the pellets were mixed with isolation buffer without EGTA (Berman and Hastings 1999).
Chapter 3

3.2.6.2. Measurement of mitochondrial enzyme complex activities

3.2.6.2.1. Complex-I (NADH dehydrogenase) activity

NADH dehydrogenase enzyme also known as NADH: ubiquinone oxidoreductase or complex-I is the largest respiratory enzyme with 44 polypeptide chains located in the inner mitochondrial membrane. Its function is to catalyse the transfer of electrons from NADH to coenzyme Q10 (Co Q10). For each single NADH, four protons will be translocated into the inner membrane aiding in electrochemical gradient. Overall 5% of electrons are being directed for superoxide formation by this enzyme.

\[
\text{NADH} + \text{H}^+ + \text{CoQ} + 4\text{H}^+_{\text{in}} \rightarrow \text{NAD}^+ + \text{CoQH}_2 + 4\text{H}^+_{\text{out}}
\]

King and Howard method (King and Howard 1967) involving oxidation of NADH to NAD\(^+\) with consecutive reduction of cytochrome C was employed to determine the complex 1 activity. Briefly, the reaction mixture contained 0.2 M glycyl glycine buffer pH 8.5, 6 mM NADH in 2 mM glycyl glycine buffer, & 10.5 mM cytochrome C. Test sample was added to the above mixture & the change in absorbance at 550 nm was recorded during a 2 min period and the results were expressed as percentage of sham.

3.2.6.2.2. Complex-II (Succinate dehydrogenase) activity

Complex-II also known as succinate dehydrogenase or succinate-coenzyme Q reductase (SQR) is attached to the inner membrane of the mitochondria. It connects the Krebs cycle and the mitochondrial ETC. SDH oxidises succinate to fumarate with subsequent transfer of electrons to ubiquinone.

Succinate dehydrogenase (SDH) activity was spectrophotometrically quantified as per the method of King (King 1967). The method utilises the oxidation of succinate by an artificial electron acceptor (potassium ferricyanide). The assay consists of mixture of 0.2 M phosphate buffer pH 7.8, 1% BSA, 0.6 M succinic acid, & 0.03 M potassium ferricyanide. The test sample was added to the above reaction mixture and the change in absorbance at 420 nm was recorded for 2 min duration immediately and the results were expressed as percentage of sham.

3.2.6.2.3. Mitochondrial redox activity (Complex-III) assay

The MTT assay is a routine laboratory test for determining the cytotoxic potential, involving the measure of the enzyme activity that reduces MTT\(^+\) (yellow) to
formazan (purple) in live cells, giving a purple color. Acidified ethanolic solution of dimethyl sulfoxide is used to dissolve the insoluble purple formazan. The absorbance of this colored solution can be quantified by measuring absorbance between 500 and 600 nm. In live/viable cells only, this active form of reductase enzyme is present and used to quantify the cell viability. Quantity of formazan formed in treated cells is compared with that of the untreated cells to deduce the cytotoxic potential.

MTT assay employed in the current study is based on the studies described by Liu and their group involving the conversion of MTT tetrazolium salt to formazan crystals by mitochondrial respiratory chain reactions. The end product’s absorbance was quantified by using an ELISA reader (Biorad-Xmerk) at a wavelength of 580 nm and the results were expressed as percentage of sham (Liu et al. 1997).

3.2.6.2.4. Complex-IV (Cytochrome oxidase activity)

Complex IV also known as cytochrome C oxidase is a large trans-membrane enzyme complex found in the mitochondrial ETC. It accepts electron from each of 4 cytochrome C molecules, and transfers them to oxygen converting one molecule of oxygen into two molecules of water.

\[
4 \text{Fe}^{2+}\text{-cytochrome } c + 8 \text{H}^+_\text{in} + \text{O}_2 \rightarrow 4 \text{Fe}^{3+}\text{-cytochrome } c + 2 \text{H}_2\text{O} + 4 \text{H}^+_\text{out}
\]

During this, it also translocates four protons across the membrane, establishing a trans-membrane proton gradient along with formation of water molecule. Cytochrome oxidase enzyme activity in spinal mitochondria was quantified according to the method of Sotocassa and their group (Sottocasa et al. 1967). The reaction mixture consists of 0.3 mM reduced cytochrome C in 75 mM phosphate buffer. Mitochondrial test sample was added to the above mixture. The change in absorbance was recorded at 550 nm over the period of 2 min immediately & the results were expressed as percentage of sham.

3.2.7. Statistical analysis

Refer to chapter 1 (1.2.8)
3.3. Results

3.3.1. Effect of pioglitazone, ceftriaxone and their combination on mechanical allodynia

No significant difference in mechanical allodynia was observed between sham and naïve group. SNL treatment significantly caused marked mechanical allodynia as evidenced by decrease in paw withdrawal threshold to vonfrey stimuli as compared to sham group.

![Mechanical Allodynia Graph](image)

**Figure 3.2 Effect of pioglitazone, ceftriaxone alone and their combination on mechanical allodynia in SNL induced neuropathic pain.** Data were expressed as mean ± S.E.M. \(^a p < 0.05\) compared to sham group; \(^b p < 0.05\) compared to SNL group; \(^c p < 0.05\) compared to Pio (5); \(^d p < 0.05\) compared to Pio (10); \(^e p < 0.05\) compared to Cef (100); (Two way ANOVA followed by Bonferroni posttests). SNL: spinal nerve ligation; Pio: pioglitazone; Cef: ceftriaxone.

Pioglitazone (10, 20 mg/kg), ceftriaxone (200 mg/kg) treatment significantly improved paw withdrawal threshold (Fig. 3.2) as compared to SNL control.
However, lower doses of both pioglitazone (5 mg/kg) and ceftriaxone (100 mg/kg) did not demonstrate any significant effect on mechanical allodynia as compared to SNL control group. Moreover, pioglitazone (5 mg/kg) in combination with ceftriaxone (100 mg/kg) showed significant improvement in paw withdrawal threshold from day 14 onwards as compared to their effect per se in SNL treated groups. Further, pioglitazone (20 mg/kg) in combination with ceftriaxone (200 mg/kg) did not show any significant improvement in paw withdrawal threshold as compared to their per se effect in SNL treated groups. Besides, per se treatment of higher doses of both pioglitazone (20 mg/kg), ceftriaxone (200 mg/kg) did not demonstrate any significant effect on mechanical allodynia parameter as compared to sham treatment (data not shown).

3.3.2. Effect of pioglitazone, ceftriaxone and their combination on mechanical hyperalgesia

No significant difference in mechanical hyperalgesia was observed between sham and naïve group. SNL caused significant mechanical hyperalgesia ($p < 0.0001$) (decreased paw withdrawal threshold) as compared to sham group. Pioglitazone (10, 20 mg/kg), ceftriaxone (200 mg/kg) treatment significantly improved paw withdrawal threshold (Fig. 3.3) as compared to SNL control. However, lower doses of both pioglitazone (5 mg/kg) and ceftriaxone (100 mg/kg) did not demonstrate any significant effect on mechanical hyperalgesia as compared to SNL control. Moreover, pioglitazone (5 mg/kg) in combination with ceftriaxone (100 mg/kg) significantly improved paw withdrawal threshold (day 7 onwards) as compared to their individual effects in SNL treated animals. Nevertheless, pioglitazone (20 mg/kg) in combination with ceftriaxone (200 mg/kg) did not demonstrate any significant effect on mechanical hyperalgesia as compared to their effects per se in SNL treated animals.
Figure 3.3 Effect of pioglitazone, ceftriaxone alone and in combination on mechanical hyperalgesia in SNL induced neuropathic pain. Data were expressed as mean ± S.E.M. $^a p < 0.05$ compared to sham group; $^b p < 0.05$ compared to SNL group; $^c p < 0.05$ compared to Pio (5); $^d p < 0.05$ compared to Pio (10); $^e p < 0.05$ compared to Cef (100); (Two way ANOVA followed by Bonferroni posttests). SNL: spinal nerve ligation; Pio: pioglitazone; Cef: Ceftriaxone.

3.3.3. Effect of pioglitazone, ceftriaxone and their combination on cold allodynia

No significant difference in cold alldodynia was observed between sham and naive group animals. SNL treatment significantly caused cold alldodynia as evidenced by significant ($p < 0.0001$) reduction in paw withdrawal latency (lifting and licking) in response to acetone treatment as compared to sham treated group. Pioglitazone (10, 20 mg/kg), ceftriaxone (200 mg/kg) treatment significantly improved paw withdrawal latency (Fig. 3.4A & 3.4B) as compared to SNL control.
Figure 3.4 Effect of pioglitazone (A), ceftriaxone (B) alone and in combination on cold alldynia in SNL induced neuropathic pain. Data were expressed as mean ± S.E.M. \(^a_p < 0.05\) compared to sham group; \(^b_p < 0.05\) compared to SNL group; \(^c_p < 0.05\) compared to Pio (5); \(^d_p < 0.05\) compared to Pio (10); \(^e_p < 0.05\) compared to Cef (100); (Two way ANOVA followed by Bonferroni posttests). SNL: spinal nerve ligation, Pio: pioglitazone, Cef: ceftriaxone.
Chapter 3

However, lower doses of both pioglitazone (5 mg/kg) and ceftriaxone (100 mg/kg) did not show any significant effect on cold allodynia as compared to SNL control. Moreover, pioglitazone (5 mg/kg) in combination with ceftriaxone (100 mg/kg) significantly potentiated their protective effect (increased paw withdrawal latency) as compared to their effect per se in SNL treated animals. Further, pioglitazone (20 mg/kg) in combination with ceftriaxone (200 mg/kg) did not show any significant effect on cold allodynia as compared to their effect per se in SNL treated animals.

3.3.4. Effect of pioglitazone, ceftriaxone and their combination on spinal cord oxidative damage (LPO, nitrite, SOD and GSH levels) in SNL treated animals

There is no significant difference in oxidative stress parameters was observed between naive and sham group animals. SNL treatment significantly increased LPO, nitrite, depleted SOD and GSH enzyme activities in spinal cord as compared to sham group. Pioglitazone (10, 20 mg/kg), ceftriaxone (200 mg/kg) treatment for 28 days significantly attenuated LPO, nitrite, restored SOD and GSH enzyme ($P < 0.001$) activities as compared to SNL control (Table 3.2). Whereas, pioglitazone (5 mg/kg), ceftriaxone (100 mg/kg) did not show any significant effect on these oxidative stress parameters as compared to SNL control. Meanwhile, pioglitazone (5 mg/kg) with combination of ceftriaxone (100 mg/kg) significantly enhanced their protective effects (antioxidant like effect) as compared to their effects per se in SNL treated animals. Further, pioglitazone (20 mg/kg) in combination with ceftriaxone (200 mg/kg) did not demonstrate any significant effect on oxidative stress parameters as compared to their effects per se in SNL treated animals which might be because of their ceiling effect.
Table 3.2 Effect of pioglitazone, ceftriaxone and their combination on oxidative damage (lipid peroxidation, superoxide dismutase (SOD), nitrite, and reduced glutathione (GSH) levels) in spinal nerve ligation induced neuropathic pain in rats

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>MDA nM/mg protein Mean ± S.E.M. (% of sham)</th>
<th>Nitrite µM/mg protein Mean ± S.E.M. (% of sham)</th>
<th>SOD U/mg Protein Mean ± S.E.M. (% of sham)</th>
<th>GSH (nM /mg protein) Mean ± S.E.M (%Sham)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>2.723 ± 0.13 (104.48)</td>
<td>29.11 ± 2.04 (96.32)</td>
<td>17.594 ± 2.08 (103.77)</td>
<td>56.63 ± 1.26 (105.55)</td>
</tr>
<tr>
<td>Sham</td>
<td>2.606 ± 0.12 (100)</td>
<td>30.22 ± 2.74 (100)</td>
<td>16.954 ± 1.39 (100)</td>
<td>53.65 ± 2.57 (100)</td>
</tr>
<tr>
<td>SNL</td>
<td>7.840 ± 0.28(^a) (300.84)</td>
<td>177.44 ± 6.35(^a) (587.13)</td>
<td>6.507 ± 0.85(^a) (38.38)</td>
<td>11.41 ± 0.57(^a) (21.28)</td>
</tr>
<tr>
<td>SNL + Pio (5)</td>
<td>6.954 ± 0.58 (266.83)</td>
<td>162.17 ± 9.17 (536.58)</td>
<td>8.135 ± 0.74 (47.98)</td>
<td>12.61 ± 1.71 (23.51)</td>
</tr>
<tr>
<td>SNL + Pio (10)</td>
<td>6.116 ± 0.17(^b) (234.70)</td>
<td>128.28 ± 4.42(^b) (424.45)</td>
<td>12.652 ± 0.80(^b) (74.63)</td>
<td>25.28 ± 2.62(^b) (47.13)</td>
</tr>
<tr>
<td>SNL + Pio (20)</td>
<td>4.540 ± 0.23(^b)(^d) (174.22)</td>
<td>91.04 ± 5.88(^b)(^d) (301.25)</td>
<td>15.405 ± 1.16(^b) (90.87)</td>
<td>32.35 ± 3.15(^b) (60.30)</td>
</tr>
<tr>
<td>SNL + Cef (100)</td>
<td>6.971 ± 0.29 (267.49)</td>
<td>167.02 ± 6.62 (552.63)</td>
<td>7.641 ± 0.59 (45.07)</td>
<td>14.01 ± 0.98 (26.11)</td>
</tr>
<tr>
<td>SNL + Cef (200)</td>
<td>4.559 ± 0.12(^b)(^e) (174.95)</td>
<td>129.94 ± 12.70(^b)(^e) (429.96)</td>
<td>13.011 ± 1.37(^b)(^e) (76.74)</td>
<td>22.742 ± 1.37(^b) (42.39)</td>
</tr>
<tr>
<td>SNL + Pio (5) + Cef (100)</td>
<td>5.508 ± 0.40(^b)(^c)(^e) (211.35)</td>
<td>113.27 ± 6.82(^b)(^c)(^e) (374.80)</td>
<td>13.391 ± 1.06(^b)(^e) (78.98)</td>
<td>23.84 ± 2.32(^b)(^c)(^e) (44.45)</td>
</tr>
<tr>
<td>SNL + Pio (20) + Cef (200)</td>
<td>4.693 ± 0.26(^b) (180.10)</td>
<td>91.60 ± 6.05(^b) (303.09)</td>
<td>15.258 ± 1.48(^b) (90.00)</td>
<td>28.19 ± 2.19(^b) (52.55)</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± S.E.M; in parenthesis percentage of sham was mentioned. \(^a\)p < 0.05 compared to sham group; \(^b\)p < 0.05 compared to SNL group; \(^c\)p < 0.05 compared to Pio (5); \(^d\)p < 0.05 compared to Pio (10); \(^e\)p < 0.05 compared to Cef (100); (one way ANOVA followed by Tukey’s test). SNL: spinal nerve ligation, Pio: pioglitazone, Cef: ceftriaxone.
3.3.5. Effect of pioglitazone, ceftriaxone and their combination on mitochondrial enzyme complex activities in spinal cord

No significant difference in mitochondrial enzyme complex activities (complex I-IV) was observed between sham and naive group animals. SNL treatment considerably \((p < 0.001)\) reduced enzyme activities in spinal cord in comparison to the sham treated group. Treatment with pioglitazone (10, 20 mg/kg), ceftriaxone (200 mg/kg) for 28 days significantly \((p < 0.05)\) restored the activities of enzyme complexes in spinal mitochondria as compared to SNL control (Fig. 3.5).

Fig. 3.5 Effect of pioglitazone, ceftriaxone alone and in combination on mitochondrial enzyme complex activities in SNL induced neuropathic pain. Data were expressed as mean ± S.E.M. \(^*p < 0.05\) compared to sham group; \(^b p < 0.05\) compared to SNL group; \(^c p < 0.05\) compared to Pio (5); \(^d p < 0.05\) compared to Pio (10); \(^e p < 0.05\) compared to Cef (100); (One way ANOVA followed by Tukey’s test). SNL: spinal nerve ligation; Pio: pioglitazone; Cef: ceftriaxone.
Whereas, pioglitazone (5 mg/kg), ceftriaxone (100 mg/kg) did not show any significant effect on these complex (I-IV) activities as compared to SNL control. Meanwhile, combination of pioglitazone (5 mg/kg) with ceftriaxone (100 mg/kg) significantly restored these enzyme complex activities as compared to their effects per se in SNL treated animals. However, pioglitazone (20 mg/kg) in combination with ceftriaxone (200 mg/kg) did not show any significant effect on these enzyme complex activities as compared to their effects per se in SNL treated animals.

3.3.6. Effect of pioglitazone, ceftriaxone and their combination on TNF-α, and caspase-3 levels

There is no significant ($p < 0.05$) difference was observed in proinflammatory and apoptotic markers between sham and naive group. SNL treatment significantly increased TNF-α, & caspase-3 levels in lumbar spinal cord as compared to sham group (Fig. 3.6 A-B).

![Graph showing TNF-α and Caspase-3 levels](image)

*Fig. 3.6 Effect of pioglitazone, ceftriaxone alone and in combination on TNF-α and caspase-3 in SNL induced neuropathic pain. Data were expressed as mean ± S.E.M. $^a p < 0.05$ compared to sham group; $^b p < 0.05$ compared to SNL group; $^c p < 0.05$ compared to Pio (5); $^d p < 0.05$ compared to Pio (10); $^e p < 0.05$ compared to Cef (100); ((One way ANOVA followed by Tukey’s test). SNL: Spinal nerve ligation, Pio: Pioglitazone, Cef: Ceftriaxone.*
Chapter 3

Treatment with pioglitazone (10 & 20 mg/kg), ceftriaxone (200 mg/kg) for 28 days significantly attenuated TNF-α & caspase-3 activities as compared to SNL control. Whereas, pioglitazone (5 mg/kg), ceftriaxone (100 mg/kg) did not show any significant effect on these TNF-α levels and caspase-3 activity as compared to SNL control. Meanwhile, pioglitazone (5 mg/kg) in combination with ceftriaxone (100 mg/kg) significantly attenuated spinal cord TNF-α levels and caspase-3 activity as compared to their effects per se in SNL treated animals. Nonetheless, combination of pioglitazone (20 mg/kg) with ceftriaxone (200 mg/kg) did not demonstrate any significant effect on these TNF-α levels and caspase-3 activity as compared to their effects per se in SNL treated animals.

3.4. Discussion

Ligation of L5 and L6 spinal nerves in this study resulted in NP like symptoms as evidenced by significant allodynia and hyperalgesia to mechanical and thermal stimuli, oxidative damage, mitochondrial dysfunction, alteration of TNF-α and caspase-3 in the lumbar spinal cord. Importantly, treatment with pioglitazone, ceftriaxone and their combination significantly attenuated these behavioral, biochemical and cellular alterations in the SNL animals. Results obtained in the current study are in close agreement with the concept of positive interaction between pioglitazone and ceftriaxone in suppression of neuroinflammatory, oxidative/nitrosative stress and apoptotic pathways in SNL induced NP in rats. SNL produces hypersensitivity to variety of mechanical and thermal stimuli resembling to those of humans (Chung et al. 2004). Besides, allodynia and hyperalgesia developed in the present study are persisted for 4 weeks. Treatment with pioglitazone, ceftriaxone and their combination for 4 weeks significantly reversed these behavioral alterations. These observations are in consistent with the earlier reports of pioglitazone in different models of NP (Morgenweck et al. 2013; Jia et al. 2013). Various immune and inflammatory mediators have been proposed for the observed effects in NP (Jia et al. 2013; De Leo and Yezierski 2001). Nerve injury activates resident macrophages, schwann cells along with glial cells thereby increases release of proinflammatory mediators. These released mediators sequentially increase the hypersensitivity of the neurons (Ren and Dubner 2010). Pioglitazone has been shown to decrease the activation of these immune and glial cells thereby hyperexcitability of the
neurons (Jia et al. 2010; Jia et al. 2013) in different NP models. Based on the results obtained in the current study it seems that pioglitazone produces its protective effect on these inflammatory mediators which can be accounted for the observed changes on the behavioral parameters. Besides, GLT-1 transporters are down regulated in nerve injury condition (Amin et al. 2012; Nicholson et al. 2014). Hence, simultaneously targeting of PPARγ and GLT-1 pathways could be beneficial in nerve injury conditions. In the present study, combination of pioglitazone with ceftriaxone showed an increased reversal of these behavioral alterations suggesting an enhanced therapeutic effect over their effect per se.

Even though pioglitazone has been studied in different NP models (Jia et al. 2013; Griggs et al. 2015) to the best of our knowledge, this is the first study to report the effect of PPARγ agonists in SNL induced NP. Besides, recent study suggests an involvement of both genomic and non-genomic mechanisms of pioglitazone induced reduction in NP (Griggs et al. 2015). This raises the possibilities that apart from targeting PPARγ alone, other non genomic pathways could be involved for the pain relief. Neuronal mitochondria are the main source of ROS generation and have been implicated in NP via spinal mechanisms (Park et al. 2006). Free radicals generated through mitochondrial respiratory chain under nerve injury will lead to oxidative-nitrosative stress and different free radical scavengers have been shown to alleviate NP (Patel et al. 2012). Further, superoxide generated in the respiratory chain reactions will lead to the development of central sensitization (Janes et al. 2012). Increased glutamate release causes hyperalgesia and ROS production in nerve injury conditions (Osikowicz et al. 2013). So, minimizing glutamate levels via GLT-1 pathway could help in reducing glutamate mediated ROS generation involved in NP. In the present study, SNL induced significant oxidative stress, mitochondrial dysfunction indicating their possible interaction in the pathogenesis of NP. This is very much consistent with other reported studies (Chowdhury et al. 2010; De Leo and Yezierski 2001), indicating that nerve injury activates the immune pathway leading to release of several free radicals.

SNL is also known to increase the expression of NOS mRNA in the spinal cord leading to an increase in NO production. This increased NO reacts with superoxide radicals to form peroxide radicals. These peroxide radicals are known
to affect DNA directly and lead to neuronal death (Arora et al. 2008). Also, in the present study, increased levels of nitrite, MDA and reduced GSH, SOD and catalase activities have been observed in SNL rats. Treatment with pioglitazone, ceftriaxone decreased LPO, nitrite and restored GSH and SOD levels in spinal cord indicating their protective effect. Persistent activation of peripheral neurons results an increased release of glutamate in the spinal and supra spinal levels leading to excitotoxicity mediated neuronal damage. Simultaneously reducing the oxidative-nitrosative stress and glutamate mediated excitotoxicity with pioglitazone and ceftriaxone combination treatment as depicted in the current study, could be advantageous in combating NP.

Nerve injury affects mitochondrial functions by affecting enzyme complexes of the electron transport chain (Park et al. 2006; Xiao and Bennett 2012). Increased ROS generation affects enzyme complex function and restoring their activities could ameliorate NP (Patel et al. 2012). In the current study, SNL has been found to be associated with diminished enzyme complex (complex I-IV) activities which were restored by treatment with pioglitazone, ceftriaxone and their combinations have been found to restore their functions.

SNL alters the function of blood-spinal cord barrier, activates astrocytes (Gordh et al. 2006) and PPARγ receptors have been known to be present in CNS and spinal cords (Moreno et al. 2004; Kiaei et al. 2005). Pioglitazone and ceftriaxone have been well known to posses better blood brain barrier permeability, it may be speculated that these drugs acts at spinal and supra spinal levels to exert their protective effects. Present study data is insufficient to explain this point at this junction. Nevertheless, present study suggests that pioglitazone, interacts positively with ceftriaxone in ameliorating NP.

SNL has been known to be associated with reduced glutamate uptake in the spinal cord region which persists for 4-6 weeks (Binns et al. 2005). Besides, pioglitazone has been shown to reduce the microglial activation and thereby, prevent the development of NP (Jia et al. 2013). Glial cells eventually have GLT-1/EAAAT2 transporters on their cell membrane. These transporters are the only mechanism available to maintain the levels of glutamate below the excitotoxic levels in extracellular spaces. Mechanisms that are going to alter the expression of this GLT-1 transporter will ultimately have significant impact on glutamate
levels in extracellular spaces. Cristina Romera’s group revealed the relation between PPARγ and GLT-1 gene in causing neuroprotection by using an in-vitro culture model (Romera et al. 2007). They further proposed that rosiglitazone increased the expression of GLT-1 transporter in oxygen-glucose deprivation induced ischemic preconditioning in rat cortical cultures. Further, their study concluded that existence of PPAR response elements in the GLT-1/EAA2 promoter region and PPARγ agonists increased GLT-1/EAA2 promoter activity. Besides, NP is known to be associated with an increase in different types of chemokines including macrophage inflammatory protein-2 (Kiguchi et al. 2012) which is known to be associated with the decrease in GLT-1 expression (Fang et al. 2012). Further, PPARγ owing to its antiinflammatory actions reduce this chemokine expression (Okada et al. 2002) that could be the possible link between PPARγ activation and increased GLT-1 expression. However, ceftriaxone is well known to exhibit neuroprotection via this increase in GLT-1 expression (Mimura et al. 2011). Supporting to above, in the present study combination of pioglitazone, ceftriaxone treatment significantly caused better protection as compared to their effects alone.

Suppression of the proinflammatory cytokine activity has been suggested to be one of the pathways involved in the analgesic activities of many PPARγ and glial inhibitors. It has also well known that glial cells by themselves express the glutamate receptors (Gallo and Ghiani 2000). Thus increased glutamate acts through these receptors and releases proinflammatory cytokines causing nerve damage (Aronica et al. 2005). Further, glial inhibitors have been shown to alter mRNA and protein expression levels of mGlu 2/3, 5 and 7 receptors (Osikowicz et al. 2009). These changes have dramatic impact on lowering glutaminergic activity and reduced pain sensation. In the present study, increased TNF-α level has been observed in lumbar spinal cord. Pioglitazone, ceftriaxone combination treatment resulted in significant decrease of these proinflammatory cytokines which is significant as compared to their effects per se. Individual treatment with pioglitazone (5 mg/kg), ceftriaxone (100 mg/kg) were not shown any significant effects on these behavioral, biochemical, cellular parameters which can be accounted for their inability to reach maximum therapeutic concentrations. Besides, combination treatment of pioglitazone (5 mg/kg), with ceftriaxone (100
mg/kg) has shown significant therapeutic effect suggesting the possible additive interaction between them. However, combination treatment of pioglitazone (20 mg/kg), with ceftriaxone (200 mg/kg) did not further increase the protective effect as compared to their effects per se which could be because of their ceiling effect. Further, studies are required to know the exact reason behind this.

Earlier, PPARγ agonists have shown to decrease oxidative stress and apoptosis through the mitochondrial stabilization which is independent of their antiinflammatory actions (Fuenzalida et al. 2007; Culman et al. 2007). We report that SNL induces decrease in mitochondrial enzyme complex activities. This change has dramatic impact in caspase mediated apoptosis of the neurons. In the present study, treatment with pioglitazone, ceftriaxone have been found to restore oxidative-nitrosative stress, mitochondrial enzyme complex activities and exerted the antiapoptotic actions further supporting the rational for combination therapy (Fig 3.7).

Besides, additive analgesic effects induced by ceftriaxone were observed with the co-administration of mGlu5 receptor blocker (Inquimbert et al. 2012), microglial inhibitor (Amin et al. 2012) raises the hope for future therapeutic option for effective pain relief using combination therapy. Development of tolerance is an issue to be addressed with beta-lactam antibiotics. Reducing the dose of ceftriaxone as in the present study by combination approach or designing the drugs which specifically affect GLT-1 could be one of the ways of overcoming this issue. Further, use of specific transport inhibitors shall be undertaken to elucidate the individual role of these inhibitors in NP.

3.5. Conclusion

Present study highlights the role of pioglitazone, ceftriaxone in SNL induced behavioral, biochemical, mitochondrial and cellular alterations. Protective effect of pioglitazone, ceftriaxone could be at least in part due to their antioxidant, anti-inflammatory, antiapoptotic like effects in addition to the effects on PPARγ and GLT-1. Combination of pioglitazone with ceftriaxone could be advantageous in treating NP. However, the complex pathophysiological process requires further studies to delineate the exact mechanism involved.
Figure 3.7 Possible targets of action of pioglitazone and ceftriaxone in SNL induced neuropathic pain.