

# SECTION 4



## MATERIAL AND METHODS

#### **4.1 Experimental Animals**

Wistar albino rats of either sex, weighing 200-250 g (procured from Punjab Agriculture University, Ludhiana) were employed in present study. They were housed in animal cages with free access to water and standard laboratory pellet chow diet. The animals with cages were kept in the departmental animal house and were exposed to normal cycles of light and dark. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and the care of the animals was carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg. No- 107/ 1999/ CPCSEA).

#### **4.2 Drugs and Chemicals**

Vincristine sulfate (Chandra Bhagat Pharma Pvt. Ltd., Mumbai) and pregabalin (Ranbaxy Research Laboratories, Gurgaon, India) were dissolved in 0.9 % normal saline. Spironolactone (R.P.G, Life Sciences, Mumbai, India) and telmisartan (Torrent Pharmaceuticals, Ahmedabad, India) were suspended in 0.5 % sodium carboxy methyl cellulose (CMC). FTS (Cayman Chemicals, Michigan, USA) and GW 5074 (Sigma-Aldrich, USA) were dissolved in 10 % dimethylsulfoxide (DMSO) diluted in 0.9 % normal saline. All the reagents used in the present study were of analytical grade. TNF- $\alpha$  assay kit was procured from RayBio Inc, USA.

#### **4.3 CCI-Induced Neuropathic Pain**

Peripheral neuropathic pain was induced in rats by chronic constriction injury (Bennett and Xie, 1988) with slight modification (Muthuraman *et al.*, 2008) using silk 4-0 suture instead of chromic gut suture as it has been documented that *per se* chromic gut suture initiates the inflammatory reactions in the sciatic nerve (Maves *et al.*, 1993). In brief, rats were deeply anesthetized with ketamine (60 mg/kg *i.p.*). The hair of the rat's lower back and thigh were shaved, and the skin was sterilized with 0.5% chlorhexidine. The skin of the lateral surface of the left thigh was incised and a cut was made directly through the biceps femoris muscle to expose the sciatic nerve and four ligatures (silk 4-0) were placed around the nerve proximal part of the trifurcation with an approximate distance of one millimeter between each ligature. The ligatures were loosely tied until a

short flick of the ipsilateral hind limb was observed. After performing nerve ligation, muscular and skin layer was immediately sutured with thread, and topical antibiotic was applied. All surgical procedures were carried out under normal sterile conditions and were performed by the same experimenter.

#### **4.4 Induction of Neuropathic Pain by Vincristine**

Peripheral neuropathic pain was induced in rats by administration of vincristine sulfate (50 µg/kg *i.p.*) for 10 consecutive days (Siau and Bennett, 2006; Muthuraman *et al.*, 2008; Kaur *et al.*, 2010).

#### **4.5 Behavioral Examination**

##### **4.5.1 Paw Cold-Allodynia (Acetone Test)**

The cold allodynia was assessed by spraying a 100 µL of acetone onto the surface of the paw (placed over a wire mesh), without touching the skin. The duration of withdrawal response was recorded in seconds. The normal animals usually do not show any response (0 s) or exhibit withdrawal followed by quick recovery (assigned arbitrary value of 0.5 s) (Decosterd and Woolf, 2000).

##### **4.5.2 Mechanical Hyperalgesia (Pin Prick Test)**

The mechanical hyperalgesia was assessed by the pinprick test as described by Erichsen and Blackburn-Munro (2002). The surface of the left hind paw was touched with the point of the bent gauge needle (at 90° to the syringe) at intensity sufficient to produce a reflex withdrawal response. The duration of withdrawal response was recorded in seconds. The normal animals usually do not show any response (0 s) or exhibit withdrawal followed by quick recovery (assigned arbitrary value of 0.5 s).

##### **4.5.3 Paw Heat-Hyperalgesia (Hot Plate Test)**

The thermal nociceptive threshold, as an index of thermal hyperalgesia, was assessed by the Eddy's hot plate, maintained at a temperature of  $52.5 \pm 2.0^{\circ}\text{C}$ . The rat was placed on the hot plate and nociceptive threshold, with respect to licking of the hind paw, was recorded in seconds. The cut-off time of 15 seconds was maintained (Jain *et al.*, 2009).

##### **4.5.4. Mechanical Allodynia (von Frey Test)**

Mechano-tactile allodynia to non-noxious mechanical stimuli was assessed as described by Chaplan *et al.*, (1994). Briefly, calibrated nylon filaments, in terms of

different bending forces, were applied to the mid plantar surface of left hind paw. The filaments were applied ten times, starting with the softest and continuing in ascending order of stiffness. A brisk withdrawal of the hind limb was considered a positive response. The criterion for the threshold value, in grams, was equal to the filament evoking a withdrawal of the paw 5 times out of 10 trials *i.e.*, 50% response.

#### **4.5.5 Spontaneous pain and assessment of foot deformation**

The rat was placed on a glass floor at room temperature and was observed for 10 minutes. The cumulative duration of the paw lifting and paw licking of the ipsilateral hind limb was measured for the assessment of spontaneous pain. The lifting and licking of the paw as a part of grooming behaviour was not taken into consideration (Dowdall *et al.*, 2005). The lifting of the left paw is a combination of ongoing pain and the animal adjusting its weight bearing.

The rat was placed on a plate and the posture of the foot was observed for its deformity. The foot deformation was scored as follows: score 0 if the paw is in normal position with fanned toes, score 1 if the toe is ventroflexed; score 2 if the paw is everted so that only the internal edge of the paw touches the floor (Nakazato-Imasato and Kurebayashi, 2009).

### **4.6 Biochemical Estimations**

The animals were sacrificed on 14<sup>th</sup> day after performing behavioral tests by high dose anesthesia and the sciatic nerve was isolated immediately. The uniformity among the different nerve samples was maintained by taking the constant weight of the respective samples. The excised sciatic nerve homogenate (10 % w/v) was prepared with 0.1 M Tris HCl buffer (pH 7.4). The tubes with homogenate were kept in ice water for 30 minutes and centrifuged at 4°C (2500 rpm, 10 min). The supernatant of homogenate was separated and employed to estimate total protein content and TNF- $\alpha$ .

#### **4.6.1 Estimation of protein content**

The protein concentration in the sciatic nerve was estimated according to the method of Lowry *et al.*, (1951) using bovine serum albumin as a standard. The proteins react with Folin-Ciocalteu reagent to give blue colored complex due to reduction of phosphomolybdate (present in Folin-Ciocalteu reagent) by tyrosine and tryptophan present in the proteins. 0.3 mL of supernatant of tissue homogenate was diluted to 1 mL.

The 100  $\mu$ L of diluted supernatant was made up to 1 mL using distilled water. To this, 5 mL of Lowry's reagent was added. The contents were mixed thoroughly and the mixture was allowed to stand for 15 min at room temperature. Then 0.5 mL of Folin-Ciocalteu reagent was added and the contents were vortexed vigorously and incubated at room temperature for 30 min. The protein content was determined spectrophotometrically at 750 nm and expressed as mg/ml of 10 % sciatic nerve homogenate.

#### Preparation of reagents

##### *Preparation of Lowry's reagent*

Lowry's reagent was freshly prepared by mixing 1% w/v copper sulphate solution, 2% w/v sodium-potassium tartarate and 2% w/v sodium carbonate in 0.1 M sodium hydroxide, in the ratio of 1:1:98.

##### *Preparation of 0.1 M sodium hydroxide solution*

0.1 M sodium hydroxide was prepared by dissolving 4 g of sodium hydroxide in distilled water and volume was made up to 1 L with distilled water.

##### *Preparation of 1% copper sulphate solution*

1% copper sulphate solution was prepared by dissolving 1 g of copper sulphate in 0.1 M sodium hydroxide and volume was made up to 100 mL with sodium hydroxide.

##### *Preparation of 2% sodium potassium tartarate solution*

2 g of sodium potassium tartarate was dissolved in 0.1 M sodium hydroxide and final volume was made up to 100 mL with the same.

##### *Preparation of 2% sodium carbonate solution*

2 g of sodium carbonate was dissolved in 0.1 M sodium hydroxide and volume was made up to 100 mL with 0.1 M sodium hydroxide.

#### **4.6.2 Estimation of TNF- $\alpha$**

The levels of TNF- $\alpha$  in the sciatic nerve homogenate were estimated by commercially available enzyme-linked immunosorbent assay (ELISA). The concentration of TNF- $\alpha$  was expressed in pg/mg of protein.

##### **Principle of estimation**

TNF- $\alpha$  present in a sample and standard is bound to the wells by an immobilized antibody. Thereafter, biotinylated anti-rat TNF-  $\alpha$  antibody is added followed by addition of horse radish peroxidase (HRP)-conjugated streptavidin. The addition of 3, 3', 5, 5'-

tetramethylbenzidine (TMB), as substrate, leads to color development and the intensity of color is in proportion to the amount of TNF- $\alpha$  bound.

### **Reagents**

1. TNF- $\alpha$  Microplate: 96 wells coated with anti-rat TNF- $\alpha$
2. Wash Buffer
3. Standard: recombinant rat TNF- $\alpha$
4. Detection Antibody TNF- $\alpha$ : biotinylated anti-rat TNF- $\alpha$
5. HRP-conjugated streptavidin
6. 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution
7. Stop Solution: sulfuric acid

### **Assay Procedure**

100  $\mu$ l of standard/sample was added to each well and incubated for 2.5 hours at room temperature. Thereafter, 100  $\mu$ l of biotinylated anti-rat TNF- $\alpha$  antibody was added to each well and incubated 1 hour at room temperature followed by addition of 100  $\mu$ l HRP-conjugated streptavidin solution and incubated for 45 minutes at room temperature. 100  $\mu$ l of substrate *i.e.*, TMB was added to each well and incubated for 30 minutes at room temperature. 50  $\mu$ l of stop solution was added to each well and the color intensity was noted at 450 nm using ELISA microplate reader.

### **4.7 Intra-theal injections**

FTS and GW 5074 were administered intra-theally in a volume of 10  $\mu$ l using a Hamilton syringe. A single injection was made into the intra-theal space between lumbar regions 5-6 with a 10 mm long 27 gauge needle. The penetrations were judged successful if there was a tail flick response (Ciruela *et al.*, 2003).

### **4.8 Experimental Protocol**

In total forty two groups, each comprising six Wistar albino rats, were employed in the present study.

#### **Group I: Normal control**

Rats were not subjected to any treatment and were kept for 14 days. The behavioral tests were performed on 1<sup>st</sup> and 14<sup>th</sup> day corresponding to spironolactone and telmisartan treated groups (IV-XXIV). For experimental groups corresponding to FTS and GW 5074 (XXV-XXXXII), the behavioral tests were performed at different time

intervals 30 min, 60 min, 120 min and 180 min on 14<sup>th</sup> day. Thereafter, the animals were sacrificed to perform biochemical estimations corresponding to spironolactone and telmisartan treated groups.

**Group II: Sham control**

Rats were subjected to surgical procedure to expose the left sciatic nerve on day 1 without any nerve ligation. Thereafter, the muscular and skin layers were sutured and rats were kept for 14 days with out any treatment. The behavioral tests were performed on 1<sup>st</sup> (before surgery) and on 14<sup>th</sup> day corresponding to spironolactone and telmisartan treated groups in CCI subjected rats (IV-XI). For experimental groups corresponding to FTS and GW 5074 in CCI model (XXV-XXXII), the behavioral tests were performed at different time intervals 30 min, 60 min, 120 min and 180 min on 14<sup>th</sup> day. Thereafter, the animals were sacrificed to perform biochemical estimations corresponding to spironolactone and telmisartan treated groups.

**Group III: CCI control**

Rats were subjected to surgical procedure to expose and ligate the left sciatic nerve on day 1 as described earlier. Thereafter, the muscular and skin layers were sutured and rats were kept for 14 days with out any treatment. The behavioral tests were performed on 1<sup>st</sup> (before surgery) and on 14<sup>th</sup> day corresponding to spironolactone and telmisartan treated groups in CCI subjected rats (IV-XI). For experimental groups corresponding to FTS and GW 5074 in CCI model (XXV-XXXII), the behavioral tests were performed at different time intervals 30 min, 60 min, 120 min and 180 min on 14<sup>th</sup> day. Thereafter, the animals were sacrificed to perform biochemical estimations corresponding to spironolactone and telmisartan treated groups.

**Group IV, V and VI: Spironolactone in CCI control**

Spironolactone (5, 10 and 20 mg/kg, *p.o.*) was administered in CCI subjected rats for 14 days, from day 1 (30 minutes prior to anesthesia for surgery) to day 14. The behavioral tests were performed on 1<sup>st</sup> (before surgery) and on 14<sup>th</sup> day. Thereafter, the animals were sacrificed to perform biochemical estimations.

**Group VII, VIII and IX: Telmisartan in CCI control**

Telmisartan (1, 2 and 5 mg/kg, *p.o.*) was administered in CCI subjected rats for 14 days, from day 1 (30 minutes prior to anesthesia for surgery) to day 14. The behavioral tests and the biochemical estimations were performed as described in group IV.

**Group X: Pregabalin in CCI control**

Pregabalin (10 mg/kg, *p.o.*) was administered in CCI subjected rats for 14 days, from day 1 (30 minutes prior to anesthesia for surgery) to day 14. The behavioral tests and the biochemical estimations were performed as described in group IV.

**Group XI: CMC in CCI control**

0.5% CMC (1ml/kg *p.o.*) was administered in CCI subjected rats for 14 days, from day 1 (30 minutes prior to anesthesia for surgery) to day 14. The behavioral tests and the biochemical estimations were performed as described in group IV.

**Group XII: Saline control**

Normal saline (1ml/kg) was administered to normal rats for 10 consecutive days. The behavioral tests were performed on 1<sup>st</sup> (before administration) and on 14<sup>th</sup> day corresponding to spironolactone and telmisartan treated groups in vincristine injected rats (XIV-XXI). For experimental groups corresponding to FTS and GW 5074 in vincristine model (XXXIII-XXXX), the behavioral tests were performed at different time intervals 30 min, 60 min, 120 min and 180 min on 14<sup>th</sup> day. Thereafter, the animals were sacrificed to perform biochemical estimations corresponding to spironolactone and telmisartan treated groups.

**Group XIII: Vincristine control**

Vincristine (50 µg/kg, *i.p.*) was injected to rats for 10 consecutive days. The behavioral tests were performed on 1<sup>st</sup> (before vincristine injection) and on 14<sup>th</sup> day corresponding to spironolactone and telmisartan treated groups in vincristine injected rats (XIV-XXI). For experimental groups corresponding to FTS and GW 5074 in vincristine model (XXXIII-XXXX), the behavioral tests were performed at different time intervals 30 min, 60 min, 120 min and 180 min on 14<sup>th</sup> day. Thereafter, the animals were sacrificed to perform biochemical estimations corresponding to spironolactone and telmisartan treated groups.

**Group XIV, XV and XVI: Spironolactone in vincristine control**

Spironolactone (5, 10 and 20 mg/kg, *p.o.*) was administered for 14 days in vincristine injected rats. The different behavioral tests were performed on 1<sup>st</sup> (before vincristine injection) and on 14<sup>th</sup> day. Thereafter, the animals were sacrificed to perform biochemical estimations.

**Group XVII, XVIII and XIX:** Telmisartan in vincristine control

Telmisartan (1, 2 and 5 mg/kg, *p.o.*) was administered for 14 days in vincristine injected rats. The different behavioral and biochemical tests were performed as described in group XIV.

**Group XX:** Pregabalin in vincristine control

Pregabalin (10 mg/kg, *p.o.*) was administered for 14 days in vincristine injected rats. The different behavioral and biochemical tests were performed as described in group XIV.

**Group XXI:** CMC in vincristine control

0.5% CMC (1 ml/kg *p.o.*) was administered for 14 days in vincristine injected rats. The different behavioral and biochemical tests were performed as described in group XIV.

**Group XXII:** Spironolactone *per se*

Spironolactone (20 mg/kg, *p.o.*) was administered in normal rats for 14 days. The different behavioral and biochemical tests were performed as described in group IV.

**Group XXIII:** Telmisartan *per se*

Telmisartan (5 mg/kg, *p.o.*) was administered in normal rats for 14 days. The different behavioral and biochemical tests were performed as described in group IV.

**Group XXIV:** Pregabalin *per se*

Pregabalin (10 mg/kg; *p.o.*) was administered for 14 consecutive days in normal rats. The different behavioral and biochemical tests were performed as described in group IV.

**Group XXV, XXVI and XXVII:** FTS in CCI control

Rats were subjected to surgical procedure to expose and ligate the left sciatic nerve on day 1 as described earlier. Thereafter, a single dose of FTS (2.5, 5 and 10 µg) was administered intra-theCALLY on 14<sup>th</sup> day and different behavioral tests were performed

at different time intervals such as 30 min, 60 min, 120 min and 180 min after its administration.

**Group XXVIII, XXIX and XXX:** GW 5074 in CCI control

Rats were subjected to surgical procedure to expose and ligate the left sciatic nerve on day 1 as described earlier. Thereafter, a single dose of GW 5074 (1 µg, 2 µg and 4 µg) was administered intra-theccally on 14<sup>th</sup> day and different behavioral tests were performed at different time intervals such as 30 min, 60 min, 120 min and 180 min after its administration.

**Group XXXI:** Pregabalin in CCI control

Rats were subjected to surgical procedure to expose and ligate the left sciatic nerve on day 1 as described earlier. Thereafter, a single dose of pregabalin (100 µg) was administered intra-theccally on 14<sup>th</sup> day and the behavioral tests were performed at different time intervals such as 30 min, 60 min, 120 min and 180 min after its administration.

**Group XXXII:** DMSO in CCI control

DMSO, a solvent for FTS and GW 5074, was administered intra-theccally on 14<sup>th</sup> day and the behavioral tests were performed at different time intervals such as 30 min, 60 min, 120 min and 180 min after its administration.

**Group XXXIII, XXXIV and XXXV:** FTS in vincristine control

A single dose of FTS (2.5, 5 and 10 µg) was administered intra-theccally on 14<sup>th</sup> day in vincristine injected rats and the behavioral tests were performed at different time intervals such as 30 min, 60 min, 120 min and 180 min after its administration.

**Group XXXVI, XXXVII and XXXVIII:** GW 5074 in vincristine control

A single dose of GW 5074 (1 µg, 2 µg and 4 µg) was administered intra-theccally on 14<sup>th</sup> day in vincristine injected rats and the behavioral tests were performed at different time intervals such as 30 min, 60 min, 120 min and 180 min after its administration.

**Group XXXIX:** Pregabalin in vincristine control

A single dose of pregabalin (100 µg) was administered intra-theccally on 14<sup>th</sup> day in vincristine injected rats and the behavioral tests were performed at different time intervals such as 30 min, 60 min, 120 min and 180 min after its administration.

**Group XXXX: DMSO in vincristine control**

DMSO (solvent) was administered intra-theically on 14<sup>th</sup> day in vincristine injected rats and the different behavioral tests were performed at different time intervals such as 30 min, 60 min, 120 min and 180 min after vehicle administration.

**Group XXXXI: FTS *per se***

A single dose of FTS (10 µg) was administered intra-theically in normal rats and the behavioral tests were performed at different time intervals such as 30 min, 60 min and 120 min after its administration.

**Group XXXXII: GW 5074 *per se***

A single of GW 5074 (4 µg) was administered intra-theically in normal rats and the behavioral tests were performed at different time intervals such as 30 min, 60 min, 120 min and 180 min after its administration.

**4.9 Statistical Analysis**

The results were expressed in mean ± S.E.M. The data of behavioral tests were analyzed using two way ANOVA, while the data of biochemical tests were analyzed using one way ANOVA followed by Bonferonni's post test using Graph pad prism Version-5.0 software. The  $P < 0.05$  was considered to be statistically significant.