

## Prolonged constriction of sciatic nerve affecting oxidative stressors & antioxidant enzymes in rat

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**Background & objective:** This study was carried out to determine the effects of reactive oxygen species in the balance between the pro-oxidant and antioxidant levels in experimental peripheral constriction injury induced by silver wire looping of sciatic nerve of rats.

**Methods:** Rats were divided into experimental group 1 (silver wire ligated) and group 2 (control, sham operated). Functional and behavioural activities were assessed by a modified Basso Beattie Bresnahan (BBB) locomotory rating scale. Mechanical pain intensity was measured with Randall and Selitto apparatus. Foot positioning, toe spread, paw withdrawal threshold and paw withdrawal latency were carried out on days 1, 3, 7, 14, 21 and 28 in rats with chronic pain. Oxidative stress markers such as malondialdehyde (MDA) and advanced oxidation protein products (AOPP) were measured along with antioxidants such as glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) on day 30 after constriction in sciatic nerve, spinal cord, dorsal root ganglion, dorsal root and ventral root.

**Results:** Significant ( $P < 0.05$ ) increase in MDA, AOPP, SOD and GPx and decrease in the GSH and catalase activities in sciatic nerve, spinal cord, dorsal root ganglion, dorsal root and ventral root were observed in experimental group rats compared to control group. There was no recovery in foot positioning and toe spread. Reduced paw withdrawal threshold and paw withdrawal latency was observed in ligated rats compared to control rats.

**Interpretation & conclusion:** Foot positioning, toe spread, paw withdrawal threshold and paw withdrawal latency with no recovery until day 30 confirmed locomotory deficits, hyperalgesia and neuronal impairment. Oxidative stress evidenced by increased MDA, AOPP and decreased GSH and catalase support the generation of reactive oxygen species in constriction model. The present experimental model for chronic pain induced by silver wire spirally coiled around sciatic nerve may be useful for future studies.

**Key words** Allodynia - glutathione - hyperalgesia - malondialdehyde - neuropathic pain - oxidative stress - superoxide dismutase

Oxidative stress plays an important role in various neurological disorders, such as ischaemia, Alzheimer's disease, multiple sclerosis, epilepsy, spinal cord injury and pain. Oxidative stress is an increase in

the reduction potential or a decrease in the reducing capacity of the cellular redox state<sup>1</sup> leading to the production of reactive oxygen species (ROS), which includes free radicals and peroxides. To counteract

free radical induced cell damage biological systems have evolved with endogenous mechanisms to protect themselves where as in pathological conditions, ROS leads to cellular damage<sup>2</sup>. The change that occurred in injured afferents is the sensitization of the nociceptive afferents that account for the characteristic symptoms of neuropathic pain such as allodynia, hyperalgesia and ongoing pain<sup>3</sup>. Blocking spontaneous activity reduced the above effects in neuropathic pain models<sup>4</sup>. Possibly peripheral nerve compression can initiate cellular changes in the primary afferent fibers that result in ectopic neural activity. Pain, a neural integrated physiological phenomenon which carries message from the injury to the spinal cord, and then from spinal cord to the brain may get disturbed due to excessive ROS<sup>5</sup> in neuropathic pain condition. ROS regulates expression of apoptotic protease-activating factor-1 (apaf-1), caspase-9 in the dorsal horn, spinal cord<sup>6</sup>. Increased ROS in dorsal horn neurons may contribute to central sensitization in neuropathic rats<sup>7</sup>.

Of the different methods<sup>8</sup> to induce peripheral neuropathy, in the present study an experimental model was developed whose pain was induced by external pure silver wire moderately constricting spiral looping around the sciatic nerve at mid thigh region. The purpose of using silver wire was to produce prolonged discharges recorded by the experimental studies made in our lab (unpublished data). The chromic gut model of Bennett and Xie<sup>9</sup> affects the epineural sheath undergoes dissolution into the nerve at the ligated site and there is recovery at day 22<sup>10</sup>.

There are no studies showing the involvement of oxidative stress generated by silver wire looping around sciatic nerve. In the present study we examined the effect of chronic pain producing the free radicals in peripheral nerve, and spinal neurons and its effect on the dysfunction of locomotor organ and alteration on pain sensation. Direct methods to evaluate the levels of free radicals are difficult due to their high reactivity, very short-life and low concentration and hence alternate approaches were used to measure their effect.

### Material & Methods

**Chemicals:** 2-Thiobarbituric acid (TBA), 1,1,3,3-tetraethoxy propane (TEP), chloramin-T, O-phthalaldehyde (OPT), N-ethylmaleimide (NEM), were obtained from the Sigma Chemicals Co. (St. Louis, MO, USA), reduced glutathione (GSH) was from Sisco Research Laboratory Pvt. (SRL) Chemicals (India), cumene hydro peroxide, pyrogallol was from

Hi-media Laboratory (Mumbai, India). All other chemicals and solutions were of analytical grade.

**Animals:** The Departmental Ethics Committee approved the safety procedures used for the animals and study protocol. Adult male albino Wistar rats obtained from the National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition, Hyderabad, Andhra Pradesh, India, with an average body weight of 200-225 g (2-3 months old) were housed in groups of 3-4 rats per cage with temperature (22-24°C), a 12-h light/12-h dark cycle and relative humidity 40-60 per cent. Rats had access to food and water *ad libitum*. The rats were acclimatized to the laboratory conditions for a week before the commencement of the experiment. Every effort was made to minimize the suffering of the animals and the number of animals used.

**Experimental design:** Experimental animals were distributed into two groups: group 1 (Control- sham operated), and group 2 (silver wire-ligated) with 10 rats in each group.

The rats selected for the experiment were anaesthetized with sodium pentobarbital (40mg/kg body wt, ip). The present neuropathic pain model was different from Bennett and Xie<sup>9</sup> models where silver wire was used. Briefly, after induction of anaesthesia, the hair around the mid thigh was shaved and the common sciatic nerve of the hind limb was isolated by blunt dissection through biceps femoris. The nerve was freed of the adhering tissue and was spirally coiled in 4 rounds by silver wire 1 mm spacing and affected nerve being 4-5 mm long. The degree of constriction was such that it could retard, but not arrest circulation through the superficial epineural vasculature. Similar dissection was performed to separate group of rats where the sciatic nerve was isolated but not spirally coiled with silver wire and used as controls.

The incision was closed in layers and externally antibiotic powder was sprinkled to prevent infection. After surgery, the rats were kept in individual cages. On day 30 of surgery the animals were sacrificed by CO<sub>2</sub> asphyxiation and sciatic nerve (5 mm of the lesion site), spinal cord (specifically lumbar region), dorsal root ganglion (DRG), dorsal and ventral root were extracted and stored at -80°C till further analyse were carried out.

Functional and behavioral activities were assessed in control and experimental groups by a modified Basso, Beattie and Bresnahan (BBB) locomotory

rating scale<sup>11</sup>. Foot (hind paw) positioning of the control and experimental rats was observed. Placing/stepping postures were measured by scale as used by Renno *et al*<sup>10</sup>. The toe spread was determined by lifting the rat by the base of the tail with the legs hanging free and simultaneously the toe spread reflex was observed using the following scale : Reflex present, and toes widely apart with free space in between = 3. Reflex weak, and toes not totally clubbed together with some space in between =2. Reflex absent, and toes clubbed together =1.

Mechanical pain intensity was measured with Randall and Selitto mechanical pain measurement apparatus (602000 TSE, USA) systems previously described by Randall and Selitto<sup>12</sup>. Incremental pressure via a wedge shaped piston was applied onto an area of 1.75 mm<sup>2</sup> of the dorsal surface of the hind paw. A cut-off pressure was 350 ponds/sec to avoid tissue injury. The mean of 5 consecutive values was taken separated by 10 min. Results were expressed as mean pressure in ponds (1 pond = 1 g).

The rats paw was submerged in ice cold water ( $4 \pm 1^{\circ}\text{C}$ ) and withdrawal time was measured. The changes in withdrawal latency of the two groups were compared. Foot positioning, toe spread, paw withdrawal threshold and latency were measured on days 1, 3, 7, 14, 21, 28 in rats with chronic pain.

The tissues were homogenized and biochemical parameters were carried out with 750 g and 25,000 g (at  $4^{\circ}\text{C}$ ) centrifugations depending on the assay<sup>13</sup>.

Lipid peroxidation was assessed indirectly by the measurement of secondary products, such as malondialdehyde (MDA) using an HPLC (Shimadzu corporation, Kyoto Japan, SPD- 20A UV-VIS detector, LC- 20AT liquid chromatography) method<sup>13</sup>.

Determination of advanced oxidation protein products (AOPP) (*i.e.*, some oxidation products with characteristic absorbance at 340 nm) was based on spectrophotometric (Shimadzu corporation, Kyoto Japan, UV- 160A) method detection<sup>14</sup> with minor modifications. 200  $\mu\text{l}$  of blood serum diluted 1:5 with PBS whereas in tissue 0.2-0.5 g/ml by Kalousova *et al*<sup>14</sup> method.

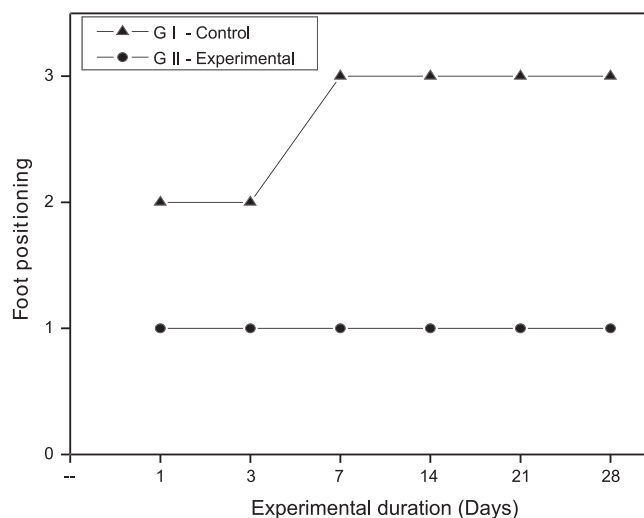
Glutathione (GSH) activity in the tissue was measured by the spectrophotometric method of Hissin and Hilf<sup>15</sup>. Superoxide dismutase (SOD) activity in the tissue was determined by spectrophotometric indirect method based on the ability of the enzyme to inhibit

$\text{O}_2^-$  dependent auto-oxidation of pyrogallol<sup>16</sup>. Catalase (CAT) activity was measured by the method of Aebi<sup>17</sup> by monitoring the disappearance of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). Glutathione peroxidase (GPx) activity in the tissue was measured by the fluorometric method of Martinez *et al*<sup>18</sup>.

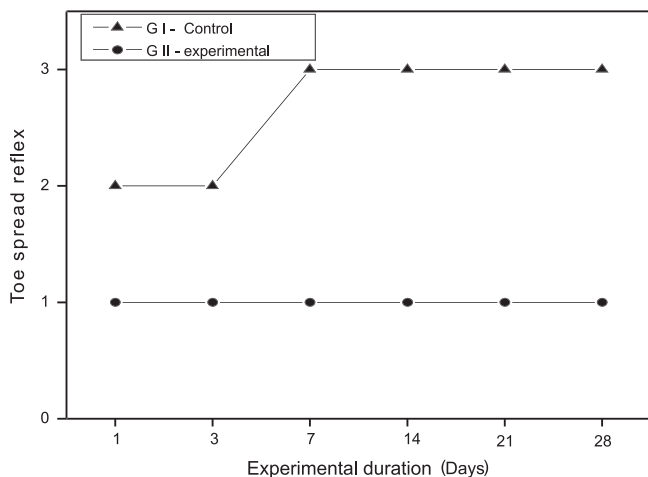
**Statistical analysis:** The differences between the control and treated groups were analyzed using Kruskal Wallis ANOVA (foot positioning and toe spread), One-way ANOVA, followed by Post Hoc test for remaining parameters.

## Results

The rats with induced neuropathy developed abnormal gait, posture, guarding behaviours, sudden licking of the hind paw *i.e.*, ipsilateral of sciatic nerve within 1-2 days after induction. The foot was ventroflexed, with the toes held tightly together and rats were unwilling to place weight on the foot of the injured side. These observations were present until day 30 in experimental rats while control rats showed normal behaviour. Foot positioning and toe spread rating was significantly different ( $P < 0.001$ ) between control and experimental groups. Both foot positioning and toe spread in group 2 rats did not show any recovery till day 30 where as in group I rats showed complete recovery on 7<sup>th</sup> day (Figs 1 & 2). Significant ( $P < 0.05$ ) reduction in paw pressure withdrawal threshold and cold allodynia in group 2 rats was observed when compared to control rats confirming reduced pain threshold levels (Figs 3 & 4), control group rats did not show any sign of recovery until 4 wk.

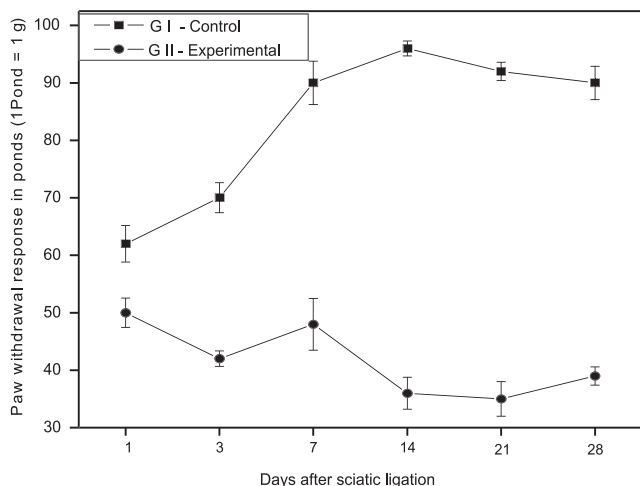


**Fig. 1.** Effect of constriction injury induced by silver wire looping on foot positioning of rats when compared to control groups. The data are the mean  $\pm$  SD ( $n=6$ ). Group-I data is significantly different from group-II at  $P < 0.05$ .

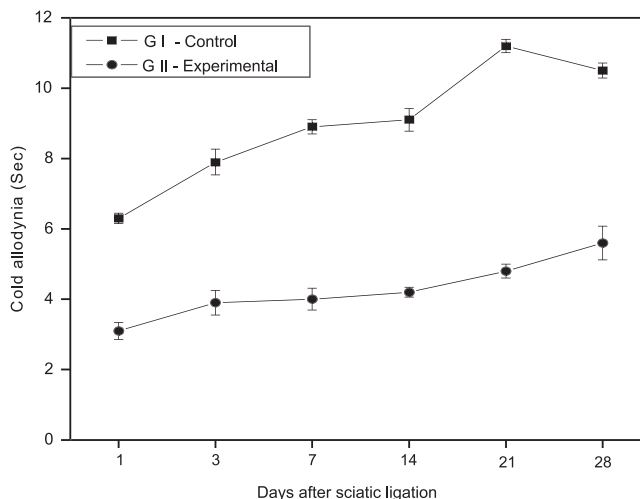


**Fig. 2.** Effect of constriction injury induced by silver wire looping on toe spread reflex of rats when compared to control groups. The data are the mean  $\pm$  SD (n=10). Group-I data is significantly different from group-II at  $P<0.05$ . Both foot positioning and toe spread reflex are performed depending on the rating scale. Though 1, 2, 3 rating is given the position varies in both the methods clearly explained in reference papers. Since both the methods are non-parametric assays KruskalWallis statistics have been performed.

Malondialdehyde and advanced oxidation protein products measured at the end of day 30 after surgery increased significantly ( $P<0.05$ ) in sciatic nerve, spinal cord, DRG, dorsal root and ventral root when compared to control rats indicating an increase in lipid peroxidation and protein oxidation. The values of radical scavenging enzymes superoxide dismutase and glutathione peroxidase increased significantly ( $P<0.05$ ) in group 2 rats when compared to group 1.



**Fig. 3.** Effect of constriction injury induced by silver wire looping on paw withdrawal threshold of rats when compared to control groups. The data are the mean  $\pm$  SD (n=6). Group-I data is significantly different from group-II at  $P<0.05$ .



**Fig. 4.** Effect of constriction injury induced by silver wire looping on cold allodynia of rats when compared to control groups. The data are the mean  $\pm$  SD (n=6). Group-I data is significantly different from group-II at  $P<0.05$ .

Reduced glutathione and catalase levels were inhibited significantly ( $P<0.05$ ) when compared to control group rats (Table).

**Discussion**

Peripheral nerve injury can be studied by electrophysiological and histological methods but functional evaluation is important to know the degree of injury and recovery. Hence the most reliable, quantitative and reproducible methods is sciatic functional index<sup>19</sup> which takes into account the relation between toes and feet of hind limbs. Foot positioning and toe spread are useful in assessing not only the locomotory and behavioural movements but also the degree of injury. Significant reduction in paw withdrawal and cold allodynia thresholds confirms nocifensive behavioural and hyperalgesic effect.

Increase in the free radical activity is the possible mechanism that is operating to modulate remarkable pathophysiological phenomenon associated with nerve injury<sup>20</sup>. Peripheral nervous system has a rich source of lipids and may be the predominant target of free radical mediated lipid peroxidation. In the present study lipid peroxidation (LPO) as clear cut marker of oxidative stress increased MDA levels significantly in group 2 rats compared to the group 1 rats. Similar result has been reported in sciatic nerve of rats induced with chronic constriction injury (CCI)<sup>2</sup>.

AOPP levels as markers of protein oxidation<sup>21</sup> increased significantly in experimental rats when compared to control rats. This increased generation of

**Table.** Effect of constriction injury induced by silver wire looping on oxidative stress markers levels and antioxidant enzyme activity when compared to control groups

Parameters	Sciatic nerve	Spinal cord	Dorsal root ganglion	Dorsal root	Ventral root
MDA (n mole/g tissue)					
G-1	1.67 ± 0.04	3.46 ± 0.14	1.72 ± 0.09	0.70 ± 0.04	0.54 ± 0.20
G-2	3.86 ± 0.01*	6.24 ± 0.16*	3.50 ± 0.30*	1.81 ± 0.07*	1.39 ± 0.11*
AOPP (µmol/mg protein)					
G1	2.22 ± 0.04	4.02 ± 0.06	1.77 ± 0.01	1.83 ± 0.12	2.04 ± 0.05
G2	4.57 ± 0.06*	7.74 ± 0.15*	3.58 ± 0.01*	3.73±0.15*	3.48 ± 0.03*
SOD (Units/min/100 mg protein)					
G1	2.53 ± 0.11	3.62 ± 0.03	1.65 ± 0.02	1.53 ± 0.13	1.48 ± 0.12
G2	4.49 ± 0.12*	5.11 ± 0.06*	3.04 ± 0.03*	2.96 ± 0.08*	2.37 ± 0.17*
CAT (µmol H <sub>2</sub> O <sub>2</sub> decomposed/ min/ mg protein)					
G1	3.44 ± 0.65	4.69 ± 0.25	2.31 ± 0.01	1.20 ± 0.03	0.91 ± 0.02
G2	1.76 ± 0.14*	2.11 ± 0.63*	1.36 ± 0.02*	0.86 ± 0.01*	0.42 ± 0.02*
GPx (µmol GSSG formed/min/mg protein)					
G1	2.19 ± 0.09	3.20 ± 0.10	2.39 ± 0.79	1.45 ± 0.02	1.26 ± 0.15
G2	4.23 ± 0.20*	5.17 ± 0.20*	3.54 ± 0.09*	1.95 ± 0.01*	1.66 ± 0.19*
GSH (n mol/g wet weight tissue)					
G1	467.19 ± 3.90	520.99 ± 6.52	343.12 ± 2.32	202.67 ± 8.78	120.17 ± 3.20
G2	232.11 ± 1.82*	408.36 ± 4.01*	190.64 ± 1.50*	144.49 ± 3.63*	109.23 ± 2.35*

G1 Control, G2 Ligated. \*P<0.05 compared to control rats. The data are mean ± SD (n=6).

MDA, malondialdehyde; AOPP, advanced oxidation protein products; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GSH, reduced glutathione

oxidative protein products is correlated with the degree of free radicals generated<sup>22</sup>. Plasma levels of AOPP are correlated with severity of malondialdehyde and levels of proinflammatory cytokines IL- 1B- TNF-α<sup>23</sup>, suggesting that AOPP acts as a mediator of oxidative stress.

SOD form the primary defense against reactive oxygen metabolites, and GPx have been shown to be important adaptive response to condition of peroxidative stress<sup>24</sup> and the increase of peroxides is mediated by SOD action. Free radical superoxides stimulate SOD thus enhancing the activity of the enzyme. In the present study, superoxide dismutase and glutathione peroxidase were increased significantly as have been demonstrated earlier<sup>2,25</sup>.

CAT protecting the cellular constituents from oxidative damage decreased significantly in ligated rats and probably could be associated with oxidative stress or decreased antioxidant defense potential. Reduced CAT activity may result in a number of deleterious effects due to the accumulation of H<sub>2</sub>O<sub>2</sub>. Our result

of CAT decrease was different from Naik *et al*<sup>2</sup> who found no change in the catalase activity.

Glutathione, an antioxidant, protects cells from toxins such as free radicals<sup>26</sup> is significantly decreased resulting in neuropathology. Prolonged depletion of GSH in the brain is associated with oxidative neuronal death<sup>27</sup>. The decrease in glutathione levels in nerve<sup>2</sup>, spinal cord<sup>28</sup>, dorsal root ganglion, dorsal root and ventral root of experimental rats represents increased susceptibility to oxidative stress and hyperalgesia. The decline in GSH content seen in the present study may be due to its consumption to challenge the prevailing oxidative stress. The differences in the oxidative stress markers and antioxidant enzymes in sciatic nerve, spinal cord, dorsal root ganglion, dorsal root and ventral root showed that all regions were not equally susceptible to oxidative damage and were neither equally protected by antioxidant defenses.

In summary, this study showed that free radical activity increased at the site of silver wire looping

and contributed to the maintenance of cold allodynia and thermal hyperalgesia. Our study also showed that the present experimental model for induction of neuropathic pain was effective in producing ROS which play an important role in neuropathic pain, predominantly through spinal mechanisms.

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