Role of Neuropeptides and Ginsenoside Rg1 in Peripheral Nerve Injury-Induced Neuropathic Pain in Mice

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Abstract

Ginsenoside Rg1 (GRg) is a natural bioactive flavonoid compound. It has potential action on the neuronal system and it prevents the neurodegenerative disorders via alteration of neuropeptides. The present study is focused on evaluating the role GRg and neuropeptides in peripheral nerve injury-induced neuropathic pain. The test compound i.e., ginsenoside Rg1 (5 and 10 mg/kg; i.v.) and reference compound i.e. pregabalin (PrG: 5 mg/kg; i.v.) were administered for 10 consecutive days. The pain sensations were assessed by various tests like acetone drop, pinprick, plantar, tail flick and tail pinch test. All behavioral tests were performed at different time intervals i.e., 0, 4, 8, 12 and 16th day. The biochemical changes like thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), superoxide anion, calcium, myeloperoxidase and tumor necrosis factor alpha (TNF-α) and neuropeptide i.e., calcitonin gene-related peptide (CGRP) levels were estimated in sciatic nerve tissue. Treatment of GRg and pregabalin attenuated the CCI induced pain response in a dose-dependent manner. Further, it also ameliorated the tissue biochemical changes. Statistically significant ($p < 0.05$) results were observed in test and reference drug-treated groups. GRg has potential neuroprotective actions against CCI induced neuropathic pain. It may attribute to multiple pharmacological actions i.e., free radical scavenging; down-regulation of TNF- α proteins; and reduction of calcium accumulation leads to produce the neuroprotective actions.

***Keywords:** Chronic Constriction Injury; Ginsenoside; Myeloperoxidase; Pregabalin; Superoxide Anion; Sciatic Nerve*

Abbreviations

ANOVA: Analysis of Variance; CCI: Chronic Constriction Injury; CGRP: Calcitonin Gene-Related Peptide; CGRP: Calcitonin Gene-Related Peptide; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals; CRPS: Complex Regional Pain Syndrome; DTNB: 5,5'-Dithiobis(2-nitrobenzoic acid); GRg: Ginsenoside Rg1; GSH: Reduced Glutathione; IAEC: Institutional Animal Ethics Committee; IL: Interleukin; MPO: Myeloperoxidase; NANC: Non Adrenergic-Noncholinergic; NBT: Nitroblue Tetrazolium; PrG: Pregabalin; SD: Standard Deviation; TBARS: Thiobarbituric Acid Reactive Substances; TNF-α: Tumor Necrosis Factor Alpha

Introduction

Neuropeptides play a key role in the regulation of the central and peripheral nervous system. Peripheral nerve injury is known to elevate the neuropeptides like bradykinin, substance P and calcitonin-gene-related peptide (CGRP). CGRP has widely employed the progress of neuropathic pain [1]. Neuropathic pain is an unpleasant emotional experience with a painful sensation. It is mainly caused by damage of the somatosensory system [2]. In addition, the pathogenesis of neuropathic pain is due to the alteration of various neuronal impulses;

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plasticity; and subsequently accelerates the neurodegenerative process [3]. The various ion channels are responsible for the alteration of the neuronal signal; synaptic plasticity; and enhancement of neuronal excitation [4]. Our previous study revealed that calcium channel alteration and accumulation of cytosolic calcium channels are contributing to the progress of neuropathic pain via activation of m-calpain and calmodulin proteins [5,6]. Moreover, the neuronal oxidative stress also develops by the calcium-dependent and independent activation of free radicals formation i.e., superoxide anion ($\cdot O_2^-$); and peroxynitrite ($\cdot ONOO$) [7]. Thus, the sequential and/or subsequent actions of free radicals synthesis; calcium accumulation; and release of TNF-α proteins are contributing to the progress of oxidative stress; neuroinflammation; and neurodegeneration [8]. Further, peroxynitrite and TNF-α are significantly contributed in the release and expression of CGRP in the nerve terminals leads to exacerbating the neuropathic pain [9]. The administration of CGRP release inhibitor (triptans, opioid) and CGRP receptor antagonist ($CGRP_{8-37}$ and CGRP-antibodies) are documented to prevent the neuropathic pain disorders [10,11]. The administrations of conventional medicines are documented to reduce the neuropathic pain symptoms in human as well as in rodents [4]. However, conventional medicine produces the unwanted side effects and sometimes it produces the life-threatening effects [12]. The various plats are reported to produce the anti-neuralgic action like *Artemisia dracunculus*; *Curcuma longa*; *Crocus sativus*; *Mitragyna speciosa*; *Nigella sativa*; and *Salvia officinalis* [13]. In addition, herbal medicines like (+)-Borneol, thymoquinone, liquiritigenin, epigallocatechin gallate [14], tocotrienol [15], lycopene and resveratrol are also documented to produce the anti-neuralgic effect.

Ginsenosides is one of the saponins type steroidal glycosides and it belongs from family of *Sapindaceae*. It is found in *Gynostemma pentaphyllum* (*Cucurbitaceae*); ginseng or red ginseng; and Panax (*Ginseng*). Ginsenoside Rb_1 is the most abundant form in *Panax quinquefolius* (American ginseng) and it showed the potent neuroprotective effects. The treatment of ginsenosides Rg_5 and Rh_3 ameliorates the scopolamine-induced memory deficits [16]; multiple sclerosis and Parkinson's disease; and ischemic stroke in human [17]. In addition, ginsenosides ameliorate the lipopolysaccharide induced elevation of CGRP in peripheral neuronal tissue [18,19]. Pregabalin (PreG) is one of the anti-epileptic drugs. It is widely used in the management of diabetic neuropathic pain. In addition, it is documented to reduce the release of CGRP from nerve terminals [20]. Therefore, PreG used as a reference drug in this research work. However, the role of ginsenosides in peripheral nerve injury-induced alteration of CGRP level and management of neuropathic pain remains to be explored. Therefore, the present study designed to evaluate the role of CGRP and ginsenoside Rg1 (GRg) in peripheral nerve injury-induced neuropathic pain in mice.

Materials and Methods
Animals

The disease-free male Swiss albino mice (20 - 25 g and age of 10 months) were used for the evaluation of GRg in CCI of sciatic nerve induced neuropathic pain in mice. All the animals were kept at standard laboratory diet, temperature (37°C) and humidity condition. A 12h light-dark cycle was maintained. The animals had free access to standard laboratory diet and water *ad libitum*. This research work was approved by the Institutional Animal Ethics Committee (IAEC; No.: ATRC/09/I4) and animal care was followed 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.

Drugs and chemicals

Ginsenoside Rg1 obtained from Pioneer Enterprise Mumbai, Maharashtra, India. N-naphthylethylenediamine; sulfanilamide; and 1,1,3,3-tetramethoxy propane were purchased from Sisco Research Laboratories, Mumbai. Thiobarbituric acid and nitro blue tetrazolium (NBT) was procured from Sigma Aldrich Mumbai. Rat TNF-alpha ELISA kit was purchased from RayBio, Inc., USA. The rest of the all other chemical reagents were obtained from S.D. Fine Chemicals, Mumbai, India.

Induction of peripheral neuropathy by chronic constriction injury of sciatic nerve

Neuropathic pain was induced by CCI of the sciatic nerve as described by Ma and Eisenach method [21] with a slight modification of Kaur., *et al*. method [22]. Briefly, mice were anesthetized with thiopental sodium (40 mg/kg, i.p.). The hair lower back of right thigh re-

gion of the mice was removed and the skin was sterilized with 0.5% w/v povidone solution. The skin and muscle layers were of the thigh region were incised and sciatic nerve was exposed. The four loose ligatures (silk thread no.: 4) were placed around the proximal portion of the sciatic nerve 1 cm from the trifurcation part. The distance of 1 mm was maintained between each ligature around the sciatic nerve. The loose ligatures were applied until the short flick response appearance in ipsilateral (same side) hind paw. After completion of the CCI procedure muscle and skin, layers were sutured with silk thread and the topical antibiotic powder was applied at once.

Experimental protocol

Seven groups were employed in the present study. Each groups comprising six Swiss albino mice (n = 6). Group I (Normal control): Mice were not subjected to any surgical procedure and kept for 10 consecutive day's normal research laboratory conditions. Group II (Sham control): Mice were subjected to expose the right sciatic nerve without any nerve ligation by standard surgical procedure. This group was providing the information for nerve injury associated neuropathic pain with a comparison of CCI control group. Group III (CCI control): Mice were subjected to expose and ligation the right sciatic nerve under anesthetic condition. The procedure was described in induction of neuropathic pain section. Group IV (GRg per se): Mice were subjected to the administration of GRg (10 mg/Kg, i.v.) for 10 consecutive days in healthy normal Swiss albino mice. Group V and VI (GRg; 5 and 10 mg/kg): Mice were subjected to the intravenous administration of GRg (5 and 10 mg/Kg) for 10 consecutive days respectively. Group VII (PreG; 5 mg/kg): Mice were subjected to the intravenous administration of PreG (5 mg/Kg) for 10 consecutive days. Each drug was administered for 10 consecutive days starting from day 1 (day of surgery). All seven groups were employed for the assessment of behavioral and biochemical evaluations. All neurobehavioural tests were performed at different time intervals i.e. 0, 4, 8, 12 and 16th day. Each day of behavioural assessment were performed based the low to high pain sensations intensity against the stimuli i.e., acetone drop, pinprick, plantar, tail flick and tail pinch test. On the 16th day, all the animals were sacrificed. The sciatic nerve and surrounding tissue samples were collected for further biochemical evaluation.

Behavioral evaluation
Acetone drop test

The cold chemical sensitivity of the right hind paw was assessed by Choi., *et al*. method [23]. It clinically resembles thermal allodynia symptoms. Briefly, the mice were placed on a wire mesh grid. The acetone (100 μl) was sprayed on the plantar surface of the right hind paw of the mice after 5 minute accommodation period. The 1-minute duration was maintained for observation of acetone induced cold sensitive reaction. The pain sensitive reactions were scored i.e. 1 for paw licking; 2 for shaking; 3 for right hind paw lifting duration less than 4 seconds; 4 for right hind paw lifting duration between 5 to 8 seconds; and 5 for right hind paw lifting duration above 8. The total score was noted as 15. Highest and lowest score depicts severe neuronal injury associated dysfunction of neuron and neuroprotection respectively.

Pin prick test

The mechanical pain sensation was assessed by Erichsen and Blackburn-Munro method [24]. Clinically, it resembles the pinpoint mechanical hyperalgesic symptoms. Briefly, the blunted needle was touched to the mid-plantar surface of the right hind paw. The intensity was generated until the detectable reflex withdrawal response in right hind paw of normal as well as neuropathic pain control animals. The needle was applied six times per minute. The cut off stimuli was applied only six times to avoid the unwanted tissue injury and development of wind-up phenomenon.

Plantar test

The radiant heat sensation was assessed in ipsilateral hind paw by Hargreaves., *et al*. method [25]. Clinically, it is mimicking the thermal hyperalgesic symptoms. Briefly, the right hind paw of mice was placed on the radiant heat lamp source. The radiant heat sensitivity of the hind paws was noted as hind paw withdrawal latency. The brisk withdrawal of the hind limb was considered a a painful response. The cut off time was maintained at 20 seconds.

Tail flick test

The radiant heat sensation was assessed in the tail part of the mice by D'Aemour and Smith method [26] with a slight modification of Hargreaves., *et al*. method [25]. Clinically, it resembles central thermal sensation symptoms. Briefly, the 1 cm distance from the tail terminal region of mice was placed on the radiant heat lamp source. The radiant heat sensitivity of the tail was observed as the tail withdrawal latency. The quick withdrawal of the tail from heat lamp source was considered a a painful response. The cut off stimuli was maintained for 15 seconds to avoid the potential tissue damage of the tail skin.

Tail pinch test

The mechanical pain sensation was assessed in the tail part of the mice by the method of Takagi., *et al*. method [27]. Clinically, it resembles central mechanical pain sensation symptom. Briefly, Hoffmann clamp was placed on the base of the tail. The screw of the Hoffmann clamp was adjusted to develop the mechanical pressure and elicit the painful sensation response within 5s. The rising number of dislodgment attempt on the clamp was noted as a painful response. The cut-off time for the application of mechanical pressure was maintained for 10 s to prevent the potential tissue damage on the mice skin.

Biochemical estimation

All the tissue samples were kept in the humidity chamber and maintained at 85% relative humidity and 37ºC. The 10% w/v of sciatic nerve homogenate was prepared with 0.1M Tris-HCl buffer (pH 7.4); deionised water; and phosphate buffer (pH 7.4) for total protein, thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH); total calcium; tumor necrosis factor-alpha (TNF-α) estimation respectively. Superoxide anion was also estimated in a sciatic nerve tissue sample. Further, surrounding muscular tissue was homogenated with phosphate buffer (pH 7.4) and used for the further estimation of myeloperoxidase (MPO) activity.

Estimation of TBARS

The thiobarbituric acid reactive substances (TBARS) was estimated by the method of Ohkawa., *et al* [28]. Briefly, 0.2 ml of supernatant of homogenate was mixed with 0.8% w/v of thiobarbituric acid and test tubes were incubated for 1h at 95ºC. The absorbance of pink color chromogen was estimated spectrophotometrically at 535 nm wavelength. A standard plot was prepared with 1 - 10 nM of 1, 1, 3, 3-tetramethoxy propane. The results of TBARS concentration were expressed as nM of MDA per mg of protein.

Estimation of reduced glutathione (GSH) content

The GSH content was estimated by the method of Beutler., *et al*. method [29]. Briefly, 0.5 ml tissue homogenate was mixed with freshly prepared 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB; 0.001 M) solution. The absorbance of yellow color chromogen was estimated spectrophotometrically at 412 nm wavelength. The standard plot was prepared with 10 - 100 µg of GSH. The results of GSH concentrations were expressed as µg of GSH per mg of protein.

Estimation of total calcium

The total calcium levels were estimated in the sciatic nerve by the method of Severnghaus and Ferrebee [30], with a slight modification of Muthuraman., *et al*. method [31]. Briefly, the sciatic nerve homogenate was mixed with 4% trichloroacetic acid and centrifuged at 1500 × g for 10 min. The supernatant was used for estimating the total calcium levels by atomic emission spectroscopy at 556 nm wavelength. The standard plot was prepared with 100-1000 parts per million (ppm) of calcium. The results of total calcium were expressed as ppm per milligram of sciatic nerve tissue.

Estimation of tumor necrosis factor-alpha (TNF-α) level

The estimation of tumor necrosis factor-alpha (TNF-α) was done measured in the sciatic nerve homogenate as described by Muthuraman., *et al*. method [5]. Briefly, recombinant anti-Rat TNF-alpha was used for the reaction development. The procedure was followed as per the instruction of commercial rat TNF-alpha ELISA kit (RayBio, Inc., USA). The TNF-α standard plot was prepared by using 0 to 20,000

pg per ml of reference standard TNF-α sample. The absorbance of yellow colored formazan was estimated spectrophotometrically at 450 nm wavelength. The results were expressed as pictograms of TNF-α per mg of total protein.

Estimation of calcitonin gene-related peptide (CGRP) level

The estimation of CGRP was carried out sciatic nerve tissue samples by using commercial CGRP - ELISA kit (RayBio Inc., USA). Briefly, recombinant anti-CGRP was used for the reaction development. The procedure was followed as per the instruction of commercial CGRP kit. The detection limit of CGRP by this ELISA kit was 4.3 pg/mL. The changes of absorbance were measured with a spectrophotometer at 450 nm wavelength. The results were expressed as pictograms of CGRP per mg of total protein.

Estimation of superoxide anion generation

The superoxide anion generation concentration was estimated by the method of Wang., *et al*. method [32], with a slight modification of Muthuraman and Singh method [33]. Briefly, the sciatic nerve was treated with 5 ml phosphate buffered saline and 100 µM of NBT and incubated at 37ºC for 90 minutes. The NBT reduction changes are reflected in the changes of color. The absorbance of purple-colored formazan was estimated spectrophotometrically at 540 nm wavelength. The quantity of NBT reduction = A x V / (T x Wt x ε x l). Where 'A' indicates the absorbance of blue formazan at 540 nm wavelength; 'V' indicates the volume of the solution; 'T' indicates the time period i.e. 90 minutes incubation with NBT; 'Wt' indicates the blotted wet weight of the sciatic nerve; 'ε' indicates the extinction coefficient of blue formazan i.e. 0.72 l per mmol per mm; and 'l' indicates the length of the light path. The results of NBT reduction were expressed as picomoles per minute per milligram wet weight of sciatic nerve.

Estimation of myeloperoxidase activity

The myeloperoxidase activity level in muscular tissue was estimated by the method of Patriarca., *et al*. method [34]. Briefly, the tissue homogenates were mixed reagent mixture i.e., DTNB to develop the yellow colored formazan. The absorbance of yellow colored formazan was estimated spectrophotometrically at 460 nm wavelength. One unit of the myeloperoxidase activity is calculated by the change of absorbance per min at pH 7.0 and 25ºC. One micromole of hydrogen peroxide used as a substrate for MPO activity assessment. The results were expressed as myeloperoxidase activity units per milligram of protein at one minute.

Estimation of total protein content

The total protein content was estimated by Lowry's., *et al*. method [35]. Briefly, tissue samples were mixed with 5 ml of Lowry's reagent and 0.5 ml of Folin-Ciocalteu reagents. The test tubes were incubated at room temperature for 30 minutes. The absorbance of purple color chromogen was estimated spectrophotometrically (UV-1800 UV-Vis spectrophotometer, SHIMADZU Corporation, Tokyo, Japan) at 750 nm wavelength. The standard plot was prepared with 1 - 10 mg of bovine serum albumin. The results of total protein concentration were expressed as mg per ml of supernatant.

Statistical analysis

All the results were expressed as mean ± standard deviation (SD). Data obtained from behavioral tests were statistically analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc analysis were applied by using Graph pad prism Version-5.0 software. The data of tissue biomarker i.e. TBARS and GSH levels were analyzed using one way ANOVA followed by Tukey's multiple range tests were applied for post-hoc analysis by using Sigmastat Version-3.5 software. A probability value of $p < 0.05$ was considered to be statistically significant.

Results
Role of GRg on CCI induced changes in acetone drop test

The performance of CCI of sciatic nerve resulted in a significant raise in thermal allodynic sensation as an indication of an increase in the scoring of chemical sensation when compared to the sham control group. Administration of GRg (5 and 10 mg/kg, i.v.) attenuated CCI

induced increase in the scoring of chemical sensation in a dose-dependent manner. The effect of GRg is comparable and similar to the pregabalin treatment group. However, vehicle and GRg (10 mg/kg; i.v.) per se treated group did not show any significant ($p < 0.05$) changes in CCI induced thermal allodynia (Figure 1).
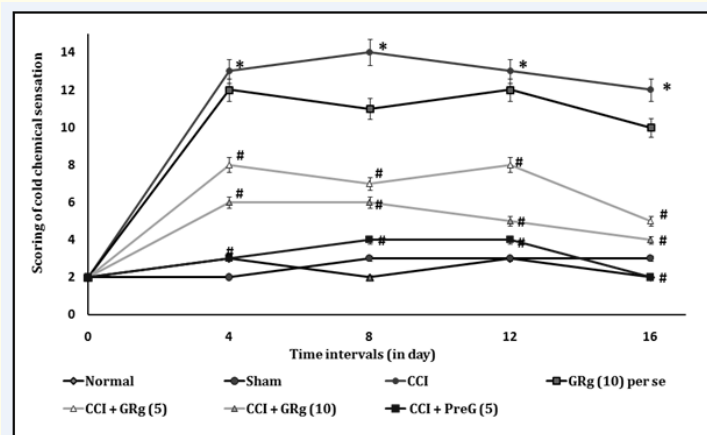

Figure 1: Role of GRg on CCI induced changes in acetone drop test (paw thermal allodynia). Digits in parenthesis indicate dose in mg/kg. Data were expressed as mean ± SD, n = 6 mice per group. *p < 0.05 Vs sham control group. #p < 0.05 Vs CCI control roup. Abbreviation: CCI: Chronic Constriction Injury and GRg: Ginsenoside Rg1.

Role of GRg on CCI induced changes in pin prick test

The performance of CCI of sciatic nerve resulted in a significant raise in mechanical hyperalgesic sensation as an indication of an increase in the percentage withdrawal of right hind paw when compared to the sham control group. Administration of GRg (5 and 10 mg/kg, i.v.) attenuated CCI induced increase in the paw withdrawal response in a dose-dependent manner. The effect of GRg is comparable and similar to the pregabalin treatment group. However, vehicle and GRg (10 mg/kg; i.v.) per se treated group did not show any significant ($p < 0.05$) changes in CCI induced mechanical hyperalgesia (Figure 2).
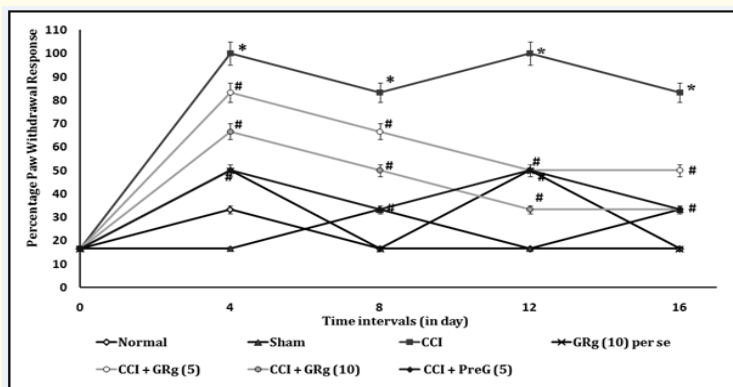

Figure 2: Role of GRg on CCI induced changes in pin prick test (paw mechanical hyperalgesia). Digits in parenthesis indicate dose in mg/kg. Data were expressed as mean ± SD, n = 6 mice per group. *p < 0.05 Vs sham control group. #p < 0.05 Vs CCI control group. Abbreviation: CCI: Chronic Constriction Injury and GRg: Ginsenoside Rg1.

Role of GRg on CCI induced changes in plantar test

The performance of CCI of sciatic nerve resulted in a significant raise in thermal hyperalgesic sensation as an indication of a decrease in right hind paw withdrawal threshold when compared to the sham control group. Administration of GRg (5 and 10 mg/kg, i.v.) attenuated CCI induced decrease in hind paw withdrawal threshold in a dose-dependent manner. The effect of GRg is comparable and similar to the pregabalin treatment group. However, vehicle and GRg (10 mg/kg; i.v.) per se treated group did not show any significant (p < 0.05) changes in CCI induced thermal hyperalgesia (Figure 3).

*Figure 3: Role of GRg on CCI induced changes in plantar test (paw heat hyperalgesia). Digits in parenthesis indicate dose in mg/kg. Data were expressed as mean ± SD, n = 6 mice per group. * p < 0.05 Vs sham control group. # p < 0.05 Vs CCI control group. Abbreviation: CCI: Chronic Constriction Injury and GRg: Ginsenoside Rg1.*

Role of GRg on CCI induced changes in tail flick test

The performance of CCI of sciatic nerve resulted in a significant raise in thermal hyperalgesic sensation as an indication of a decrease in tail withdrawal threshold when compared to the sham control group. Administration of GRg (5 and 10 mg/kg, i.v.) attenuated CCI induced decrease in tail withdrawal threshold in a dose-dependent manner. The effect of GRg is comparable and similar to the pregabalin treatment group. However, vehicle and GRg (10 mg/kg; i.v.) per se treated group did not show any significant (p < 0.05) changes in CCI induced thermal hyperalgesia (Figure 4).

Role of GRg on CCI induced changes in tail pinch test

The performance of CCI of sciatic nerve resulted in a significant raise in mechanical hyperalgesia as an indication of an increase in the number of dislodgement of Heffner's clamp from the tail when compared to the sham control group. Administration of GRg (5 and 10 mg/kg, i.v.) attenuated CCI induced increase in mechanical nociceptive pain threshold in a dose-dependent manner. The effect of GRg is comparable and similar to the pregabalin treatment group. However, vehicle and GRg (10 mg/kg; i.v.) per se treated group did not show any significant (p < 0.05) changes in CCI induced mechanical hyperalgesia (Figure 5).

Role of GRg on CCI induced changes in tissue biomarker changes

The performance of CCI of sciatic nerve resulted in a significant increase in TBARS, total calcium, TNF-α, CGRP, superoxide anion and MPO levels; and decrease in GSH content as an indication of oxidative stress, inflammation and neuronal damage when compared to the
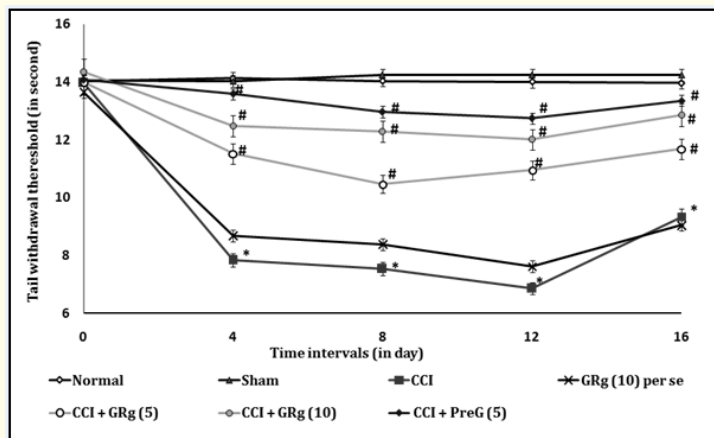
Figure 4: *Role of GRg on CCI induced changes in tail flick test (tail heat hyperalgesia). Digits in parenthesis indicate dose in mg/kg. Data were expressed as mean ± SD, n = 6 mice per group. *p < 0.05 Vs sham control group. #p < 0.05 Vs CCI control group. Abbreviation: CCI: Chronic Constriction Injury and GRg: Ginsenoside Rg1.*

Figure 5: *Role of GRg on CCI induced changes in tail pinch test (tail mechanical hyperalgesia). Digits in parenthesis indicate dose in mg/kg. Data were expressed as mean ± SD, n = 6 mice per group. *p < 0.05 Vs sham control group. #p < 0.05 Vs CCI control group. Abbreviation: CCI: Chronic Constriction Injury and GRg: Ginsenoside Rg1.*
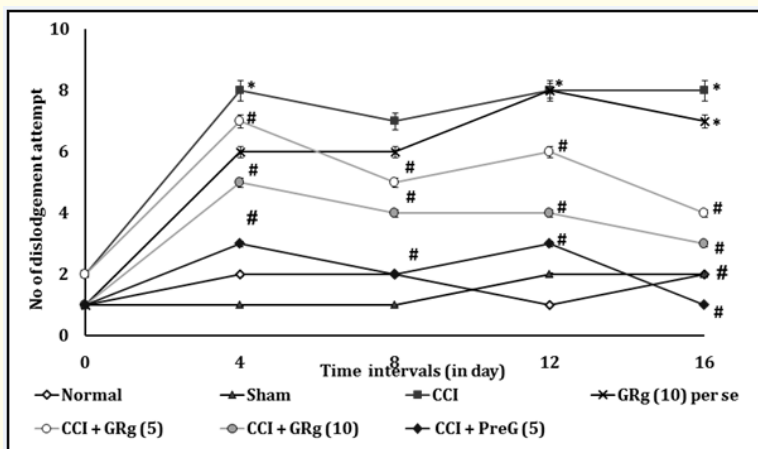
sham control group. Administration of GRg (5 and 10 mg/kg, i.v.) attenuated CCI induced changes of the above tissue biomarkers in a dose-dependent manner. The effect of GRg is comparable and similar to the pregabalin treatment group. However, vehicle and GRg (10 mg/kg; i.v.) per se treated group did not show any significant (p < 0.05) changes in CCI induced tissue biomarker changes (Table 1 and Table 2).

Groups	TBARS (nM/mg of protein)	GSH (µg/mg of protein)	Total calcium (ppm/mg of protein)	TNF-α (pg/mg of protein)	CGRP (pg/mg of protein)
Normal	2.98 ± 0.79	76.92 ± 2.05	3.03 ± 0.51	29.41 ± 0.29	35.19 ± 0.18
Sham	3.06 ± 0.48	75.38 ± 2.23	2.96 ± 0.73	43.09 ± 0.19	38.53 ± 0.68
CCI	7.94 ± 0.86*	35.65 ± 1.89*	23.94 ± 0.47*	74.04 ± 0.46*	102.79 ± 0.52*
GRg [10] *per se*	3.11 ± 0.74	74.97 ± 1.86	3.15 ± 0.69	31.79 ± 0.17	99.36 ± 0.29
CCI + GRg [5]	4.96 ± 0.62[#]	68.83 ± 2.14[#]	12.47 ± 0.38[#]	42.93 ± 0.22[#]	49.61 ± 0.39[#]
CCI + GRg [10]	4.27 ± 0.57[#]	73.75 ± 2.05[#]	5.62 ± 0.71[#]	36.76 ± 0.38[#]	40.49 ± 0.67[#]
CCI + PreG [5]	3.51 ± 0.93[#]	74.68 ± 1.94[#]	3.97 ± 0.65[#]	32.49 ± 0.61[#]	39.91 ± 0.42[#]

Table 1: *Role of GRg on CCI induced biomarker changes in tissue supernatant.*

Role of GRg on CCI induced biomarker changes in tissue supernatant. Digits in parenthesis indicate dose in mg/kg. Data were expressed as mean ± SD, n=6 mice per group. *p < 0.05 Vs sham control group. #p < 0.05 Vs CCI control group. Abbreviation: CCI: Chronic Constriction Injury; CGRP: Calcitonin Gene-Related Peptide; GRg: Ginsenoside Rg1; TBARS: Thiobarbituric Acid Reactive Substances; GSH: Reduced Glutathione.

Groups	NBT reduction (pM/Min/mg of tissue)	MPO (unit/Min/mg of protein)
Normal	2.87 ± 1.04	11.63 ± 1.83
Sham	3.13 ± 1.23	12.04 ± 1.49
CCI	24.73 ± 0.97*	137.72 ± 2.52*
GRg [10] *per se*	2.92 ± 1.04	14.27 ± 1.37
CCI + GRg [5]	9.64 ± 1.09[#]	53.91 ± 3.07[#]
CCI + GRg [10]	4.59 ± 1.11[#]	31.79 ± 2.85[#]
CCI + PreG [5]	4.01 ± 0.93[#]	17.58 ± 1.94[#]

Table 2: *Role of GRg on CCI induced biomarker changes in tissue.*

Role of GRg on CCI induced biomarker changes in tissue. Digits in parenthesis indicate dose in mg/kg. Data were expressed as mean ± SD, n=6 mice per group. *p < 0.05 Vs sham control group. #p < 0.05 Vs CCI control group. Abbreviation: CCI: Chronic Constriction Injury; GRg: Ginsenoside Rg1; NBT: Nitroblue Tetrazolium and MPO: Myeloperoxidase.

Discussion

In the present study results revealed that administration of natural medicines i.e. ginsenoside Rb1 (5 and 10 mg/Kg, i.v.) attenuated the CCI induced pain behavior thermal and mechanical hyperalgesia and allodynia in paw and tail region. It indicates that ginsenoside Rb1 possess the potential pain preventive action via neuroprotection. In addition, it also reduces the TBARS, total calcium, TNF-α, CGRP, NBT reduction and MPO activity levels; along with raising the reduced glutathione level. The chronic constriction injury model is a widely used model for the testing of mononeuritic neuropathic pain. Clinically, it mimics the complex regional pain syndrome (CRPS) with the involvement of multiple pathophysiological mechanisms in human as well as in animals [36]. The peripheral nerve injury also accelerates the neuronal signal in a different region like peripheral nerve, spinal cord and brain. In addition, various neurochemicals such as neurotransmitters i.e. serotonin, histamine and nor-adrenaline; ions i.e. sodium (Na^{2+}), potassium (K^+) and calcium (Ca^{2+}); pro-inflammatory mediators i.e. TNF-α and interleukin (IL); and free radicals like superoxide anion, peroxy nitrite and hypochlorous radicals; neuropeptides

i.e. substance P and CGRP are contributes in the pathogenesis of neuropathic pain [1,37]. TNF-α and CGRP are widely contributed to the progress of neuropathic pain at multiple levels of the nervous system i.e. nerve terminals, dorsal root ganglion, brain and blood circulation [38,39]. Furthermore, it also alters the sympathetic; para-sympathetic and non adrenergic-noncholinergic (NANC) neuronal systems [40]. Then, it alters the function of the peripheral vascular system including nerve blood barriers and neurovascular units. Subsequently, it enhances the ischemic environment for the peripheral nerve and it also enhances the accumulation of free radicals and neuroinflammation leads to aggravates the neurodegeneration [41]. The rising of free radicals known to cause the peroxidation of cellular membrane lipids and alteration of ion channels/exchangers [31]. Furthermore, the chronic activation of ion channels opening enhances the free cytosolic calcium ion (Ca^{2+}) accumulation [41,42]. Then, it is inducing the mitochondrial and nuclear DNA dysfunction via activation m-calpain proteins and apoptotic proteins and cytokines including TNF-α protein [43]. Among all, the most vulnerable molecules for the neurodegeneration and neuronal death associated neuropathic pain is m-calpain and TNF-α proteins [44]. Whereas, CGRP proteins expression are enhanced sensitization of nerve terminals [45].

The various natural plants are documented to produce the anti-neuralgic effects via scavenging of free radicals; reduction of lipid peroxidation; decreasing of inflammatory mediator synthesis; and alteration oxidative stress-related enzyme action like MPO, xanthine oxidase, glutathione reductase and glutathione peroxidase enzymes [42,46]. Our previous research report also evidenced that, plant extract of various plant i.e. *Acorus Calamus* [5], *Butea monosperma* [47], *Swietenia mahagoni* [42], *Ocimum sanctum* [36] and *Vernonia cinerea* [48]; and phytoconstituents like cannabinoids; puerarin; bulleyaconitine A; epigallocatechin gallate; tocotrienol; lycopene and resveratrol are also documented to produce the anti-neuralgic effect [13]. In the present study, ginsenoside Rb1 and pregabalin ameliorates CCI induced neuropathic pain symptoms via reduction TBARS, calcium; TNF-α, CGRP, superoxide anion and MPO levels; along with rasing the reduced glutathione level. Experimentally, ginsenoside reduced the CGRP, free radicals, oxidative stress, neuroinflammation, cytokine production and neuronal death [49,50]. The present data and literature report reveals that, ginsenoside contributes to the potential neuroprotective action against the peripheral nerve injury.

Conclusion

Ginsenoside Rb1 has the potential ameliorating effect in peripheral nerve injury-induced neuropathic pain via reduction of neuronal sensitivity, anti-oxidant; anti-inflammatory; anti-cytokines and maintenance of cytosolic calcium ion concentration. Therefore, ginsenoside Rb1 is one of the newer molecules for the reduction of neuronal CGRP and management of neuropathic pain disorders.

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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