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ATORVASTATIN POTENTIATES THE ANTI-NOCICEPTIVE EFFECT OF MILNACIPRAN IN CARRAGEENAN-INDUCED INFLAMMATORY PAIN MODEL IN RATS

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ABSTRACT

5-Hydroxytryptamine (serotonin) (5-HT) and norepinephrine (NE) are implicated in modulating descending inhibitory pain pathways in the central nervous system. Milnacipran (MLN) is a selective and potent dual 5-HT and NE reuptake inhibitor (SNRI). In the present study, the effects of HMG-CoA reductase inhibitor atorvastatin (ATR) on antidepressant- induced anti-nociception had been investigated. Anti-nociceptive and anti-inflammatory effects were evaluated in carrageenan induced inflammation and hyperalgesia in rats after administration of atorvastatin and milnacipran alone as well as in combination. Intra-plantar injection of 100 µL of 2% carrageenan produced significant rise in paw volume and reduction in paw withdrawal latency to thermal stimuli. The maximum inflammation and hyperalgesia (p>0.001) was produced at 4hr after carrageenan injection. Milnacipran did not exhibited any anti-inflammatory activity as it did not inhibit the rise of paw volume but produced statistically significant anti-nociceptive effect in this model by reducing the mean difference of paw withdrawal latency to thermal stimuli. Atorvastatin produced significant anti-inflammatory and anti-nociceptive effect. All four combinations of atorvastatin and milnacipran showed statistically significant reduction of rise of paw volume and mean difference of paw withdrawal latency. Atorvastatin modulated the antidepressant-induced anti-nociception in the model of carrageenan-induced inflammation and hyperalgesia however milnacipran failed to potentiate the anti-inflammatory activity of atorvastatin.

Key Words:- Atorvastatin, Carrageenan, Inflammation, Milnacipran.

INTRODUCTION

Pain, a complex neurobiological state, involves various neurochemical factors affecting peripheral and central pain-signaling mechanisms. Different guidelines showed that antidepressant agents are the first line treatment for neuropathic pain by varied mechanisms (Sawynok *et al.*, 2001). However, neurochemical mechanisms of anti-nociceptive effects of antidepressants have not been well described. Most antidepressants increases the monoamine levels at neuronal terminals by inhibiting the reuptake of monoamines, including norepinephrine and serotonin (Richelson and Pfenning, 1984). It is presumed that higher levels of monoamines in synaptic clefts can induce anti-nociceptive effect through changes in pain threshold. However, there is still controversy over the identity of the monoamine receptors (or receptor subtypes) responsible for these analgesic effects, in addition to their location (central or peripheral) (Korzeniewska-Rybicka and Plaznik, 1998).

It is proven that statins have various pleiotropic effects like anti-inflammatory (Wulf 2001), improvement of endothelial dysfunction (Laufs *et al.*, 1998), immunomodulatory (Niwa *et al.*, 199) and plaque stability

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(Tandon *et al.*, 2005). Kwak *et al.* (2000) showed that statins directly inhibit the induction of Major histocompatibility complex class II (MHC-II) expression by inhibiting interferon-γ (IFN- γ) and thus repress MHC-II mediated T cell activation. Various mechanisms are involved in anti-inflammatory properties of statins that may or may not involve the HMG-CoA reductase/ mevalonate pathway. Atorvastatin has pro-inflammatory as well as anti-inflammatory effects that are mainly independent of its effects on blood cholesterol (Schonbeck and Libby, 2004). Statins inhibit inflammatory responses in different models of autoimmune disease such as collagen- and complete Freund's adjuvant (CFA)-induced arthritis and experimental encephalomyelitis (Aktas *et al.*, 2003; Barsante *et al.*, 2005). Atorvastatin has shown the anti-nociceptive activity in rat model of CFA induced arthritis (Barsante *et al.*, 2005).

Taking into account the anti-inflammatory and anti-nociceptive properties of antidepressants and statins in animal model of pain and inflammation, the aim of this study was to investigate whether atorvastatin potentiate the anti-nociceptive effect of milnacipran or milnacipran potentiate the anti-inflammatory activity of atorvastatin.

MATERIAL AND METHODS

Drugs and Chemicals

Atorvastatin and milnacipran were kind gift from ONS Pharma, Rajasthan, India. Atorvastatin and milnacipran suspensions were prepared in 0.5% methyl cellulose in water for this study. Lambda (λ)-carrageenan was purchased from Sigma-Aldrich, St. Louis, MO and 2% solution was prepared in sterile 0.9% normal saline. Chemical like methyl cellulose powder used for the study was of analytical grade.

Animals

Male Sprague-Dawley rats, 112 in number, weighing 190 to 250g were used in this study. The rats were housed in groups of four animals at 20 to 25°C, in a humidity-controlled room under a 12:12-h light/dark cycle. The rats were supplied food and water *ad libitum* and were given at least 3 days to adapt to the animal room before being tested. The animals were brought to the test room at least 1 h before testing. Each rat was used for only one experiment. All procedures were approved by our Institutional Animal Ethics Committee.

Induction of unilateral inflammation and hyperalgesia

The method of Tz-Chong Chou *et al.*, 2003 was used for this experiment with some modifications. To induce local inflammation, 2 mg of λ-carrageenan (Sigma, St. Louis, MO; 100 µL of 2% w/v in saline) was injected subcutaneously into the plantar surface by intra - plantar injection (i.pl.) of rat right hind paw.

Experimental design

The study was performed in four sets of experiments. In all experimental sets, each group contains 8 rats. First set of experiment comprised of the time course evaluation of vehicle and 2% carrageenan treated rats. One group of animals was treated with vehicle alone i.e. 0.5% methyl cellulose in water in each experiment. The treatment was given to the animals of respective group one hour prior to inflammation and hyperalgesia assessment i.e. 3 hr after carrageenan injection. In next two experiments, animals were dosed orally either with milnacipran alone (10, 20, 40 and 60 mg/kg) or with atorvastatin alone (1, 5, 10 and 20 mg/kg). From these two experiments, two doses of each milnacipran (20 and 40 mg/kg) and atorvastatin (5 and 10 mg/kg) were selected for the last set of experiment in which animals were dosed orally with four possible combinations of milnacipran and atorvastatin.

Inflammation (paw edema) assessment

Hind paw volume was determined using a Digital Plethysmometer (model LE7500, Panlab, Spain). Right hind paw of all rats were marked with ink at the level of the lateral malleolus and baseline values for paw volume were taken before injecting carrageenan. The paw was immersed in the chamber of plethysmometer filled with water. Paw volume was determined once for each rat at each time point.

Thermal hyperalgesia assessment

The thermal nociceptive threshold was measured with a device (Plantar Test®, IITC Inc. Life Science, Woodland Hills, CA) using a method similar to that reported previously by Hargreaves *et al.*, 1988. The rats were placed in individual plastic boxes on the glass surface of the apparatus and were allowed to acclimate for 30 min before testing. The temperature of the glass surface was maintained constant at 30±01°C. A mobile radiant heat source located under the glass was focused onto the hind paw of each rat. The paw withdrawal latency was recorded by a digital timer.

For each test, five measurements were recorded, arranged in ascending order and first and last measurements were not included in the calculation. Thus, each test was calculated as a mean of three measurements and used as the thermal threshold. The cutoff of 20 s was used to prevent potential tissue damage.

Statistical analysis

All data are expressed as mean±SEM. In carrageenan alone treated rats, changes in rise in paw volume and PWL were analyzed using *Student's Paired t-test*. The statistical significance of differences between experimental data in other experiments of changes in rise in paw volume and PWL was analyzed with One-way analysis of variance (ANOVA), followed by the Tukey's post hoc test. Values of $p < 0.05$ were considered indicative of statistical significance.

RESULTS

Time course effect of carrageenan to induce inflammation and hyperalgesia

Animals received vehicle did not show any significant rise in paw volume. Rats challenged with 2% carrageenan showed significant rise in paw volume till 4 hr as compared to vehicle treated rats. After 4 hr of carrageenan injection, there was decline in the rise of paw volume (Figure 1).

Immediate after measuring paw volume, animals were evaluated for the thermal hyperalgesia (as a marker of inflammatory pain) induced after carrageenan injection using plantar test. There was statistically significant reduction in paw withdrawal latency (PWL) in vehicle treated animals as compared to 2% carrageenan treated animals starting from 1 hr to 24 hr after carrageenan injection. The maximum reduction in PWL was observed at 4 hr which in turn showing maximum thermal hyperalgesia at this time point post-carrageenan injection as shown in Figure 2.

Effect of milnacipran on carrageenan–induced inflammation and hyperalgesia

Mean % rise in paw volume were found to be 74.51 ±5.20, 68.75 ±4.09, 63.78 ±3.30 and 63.21 ±5.33 in milnacipran treatment groups at 10, 20, 40 and 60 mg/kg dose levels, respectively as shown in Figure 3 a. Milnacipran treatment did not showed inhibition of rise in paw volume in this model when compared with vehicle treated control group. Mean difference in paw withdrawal latency (PWL) were found to be 3.91 ±0.79, 2.38 ±0.43, 1.67 ±0.43 and 1.31 ±0.38 in milnacipran treatment groups at 10, 20, 40 and 60 mg/kg dose levels, respectively. Milnacipran treatment showed dose-dependent inhibition of mean difference in PWL in this model of acute inflammatory pain. There was a statistically significant reduction in the mean difference in PWL observed in 20 mg/kg ($p<0.05$), 40 mg/kg ($p<0.01$) and 60 mg/kg ($p<0.001$) when compared with vehicle treated control group as shown in Figure 3 b. The inhibitory effect of milnacipran on PWL is shown in Table 1.

Effect of atorvastatin on carrageenan–induced inflammation and hyperalgesia

Mean % rise in paw volume were found to be 67.31 ±3.59, 53.68 ±2.23, 23.06 ±2.21 and 19.61 ±1.25 in atorvastatin treatment groups at 1, 5, 10 and 20 mg/kg dose levels, respectively as shown in Figure 4 a. Atorvastatin treatment showed dose-dependent inhibition of rise in paw volume in this model of acute inflammation. There was a statistically significant reduction in the % rise in paw volume observed in 5 mg/kg ($p<0.01$), 10 and 20 mg/kg ($p<0.001$) when compared with vehicle treated control group as shown in Figure 4a. Mean difference in paw withdrawal latency (PWL) were found to be 4.46 ±0.54, 3.83 ±0.15, 3.13 ±0.27 and 3.07 ±0.21 in atorvastatin treatment groups at 1, 5, 10 and 20 mg/kg dose levels, respectively. Atorvastatin treatment did not showed dose-dependent inhibition of mean difference in PWL. There was a statistically significant reduction in the mean difference in PWL observed in 10 and 20 mg/kg ($p<0.05$) when compared with vehicle treated control group as shown in Figure 4 b. The inhibitory effect of atorvastatin on paw volume as well as on PWL is shown in Table 1.

Effect of combination of milnacipran and atorvastatin on carrageenan–induced inflammation and hyperalgesia

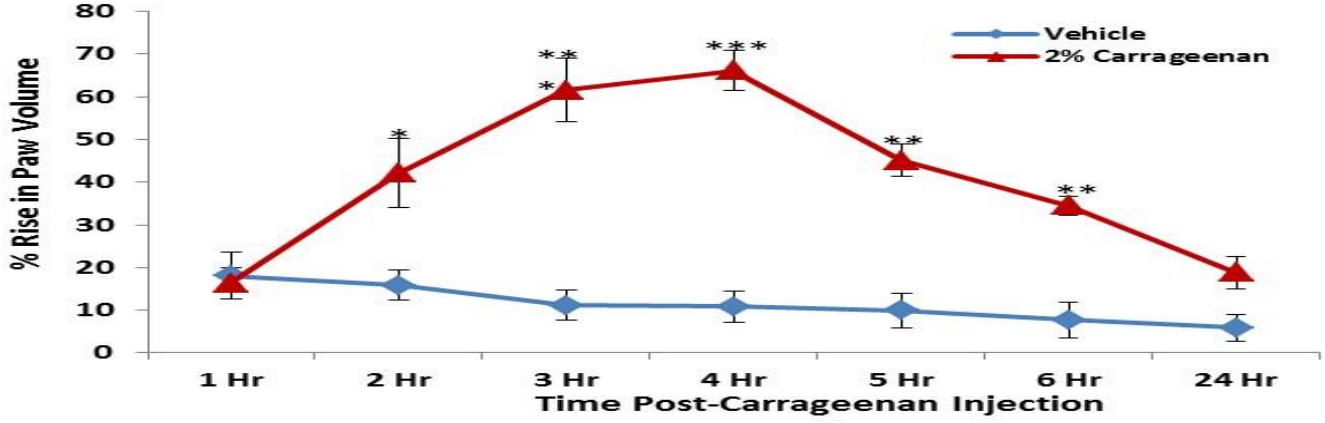
For this study two doses of milnacipran (20 and 40 mg/kg) and two doses of atorvastatin (5 and 10 mg/kg) were selected to evaluate four possible combinations for their anti-inflammatory and anti-nociceptive activity. The groups were coded as Comb-1 (MLN-20+ATR-5), Comb-2 (MLN-40+ATR-5), Comb-3 (MLN-20+ATR-10) and Comb-4 (MLN-40+ATR-10). Mean % rise in paw volume were found to be 50.05 ±4.29, 50.04 ±4.12, 19.96 ±1.70 and 21.52 ±3.79 in Comb-1, Comb-2, Comb-3 and Comb-4 treatment groups, respectively. There was a statistically significant reduction in the % rise in paw volume observed in Comb-1, Comb-2 ($p<0.01$), Comb-3 and Comb-4 ($p<0.001$) when compared with vehicle treated control group as shown in Figure 5a.

Mean difference in paw withdrawal latency (PWL) were found to be 1.99 ±0.38, 1.82 ±0.36, 1.27 ±0.16 and 1.05 ±0.28 in Comb-1, Comb-2, Comb-3 and Comb-4 treatment groups, respectively. There was a statistically significant reduction in the mean difference in PWL observed in Comb-1, Comb-2, Comb-3 and Comb-4 ($p<0.001$) when compared with vehicle treated control group as shown in Figure 5b. The inhibitory effect of all combination groups on paw volume as well as on PWL is shown in Table 1.

Table 1. Effect of atorvastatin, milnacipran and combination of atorvastatin and milnacipran in carrageenan-induced inflammation and hyperalgesia

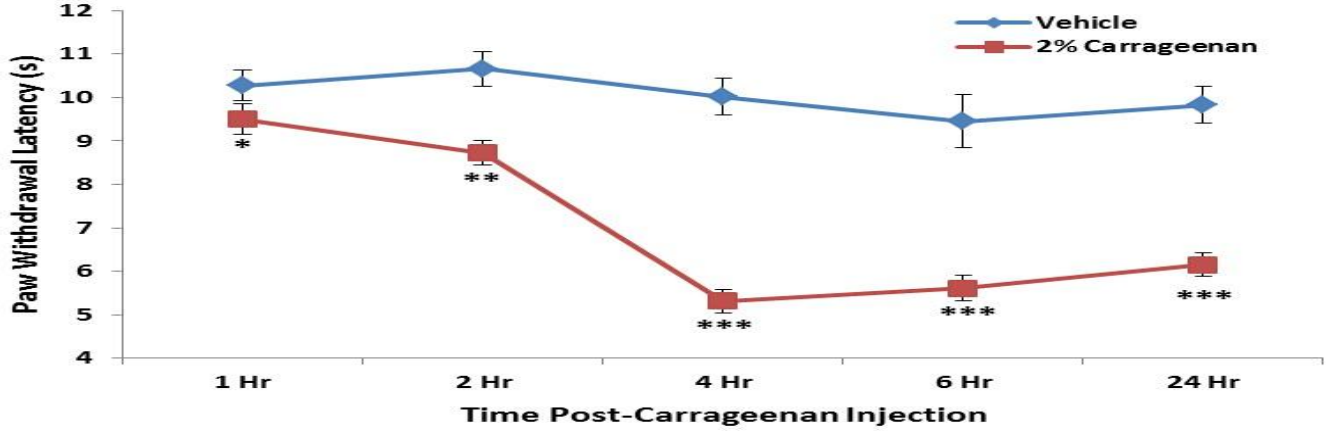
Groups		% Inhibition	
Treatment	**Dose(mg/kg)**	**% Rise in Paw Volume**	**Paw withdrawal Latency**
Milnacipran	20	-0.46	51.39*
	40	6.80	65.84**
	60	7.64	73.16***
Atorvastatin	5	21.57**	21.60
	10	66.30***	35.94*
	20	71.35***	37.29*
Milnacipran + Atorvastatin	20 + 5	26.87**	59.27***
	40 + 5	26.88**	74.05***
	20 +10	70.83***	62.85***
	40 + 10	68.55***	78.60***

Fig 1. Time course effect of vehicle and 2% carrageenan on paw volume



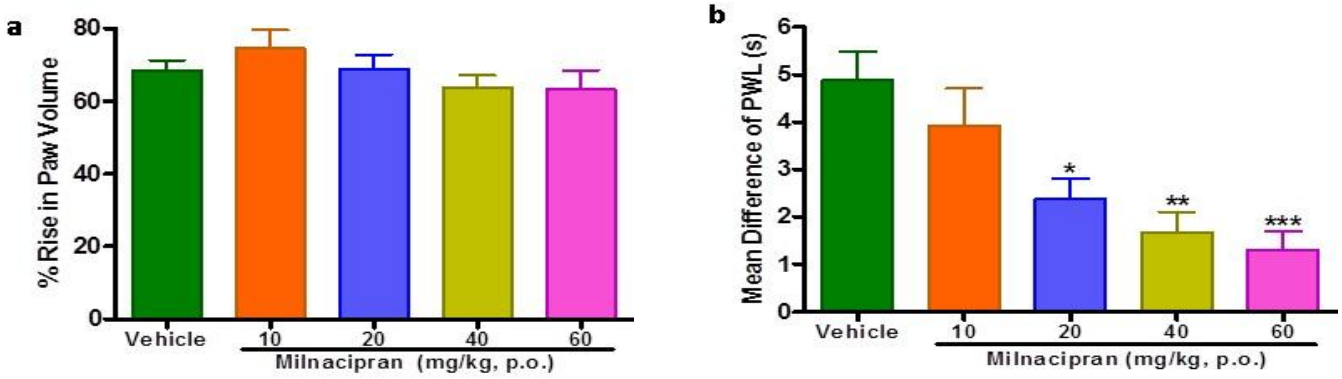
Graph shows the mean±SEM of the % rise in paw volume. *p<0.05; **p<0.01; ***p<0.001; paired *student's t-test* comparing PWL of vehicle v/s 2% carrageenan treated group at same time point.

Fig 2. Time course effect of plantar injection of 2% carrageenan on thermal hyperalgesia (paw withdrawal latency)



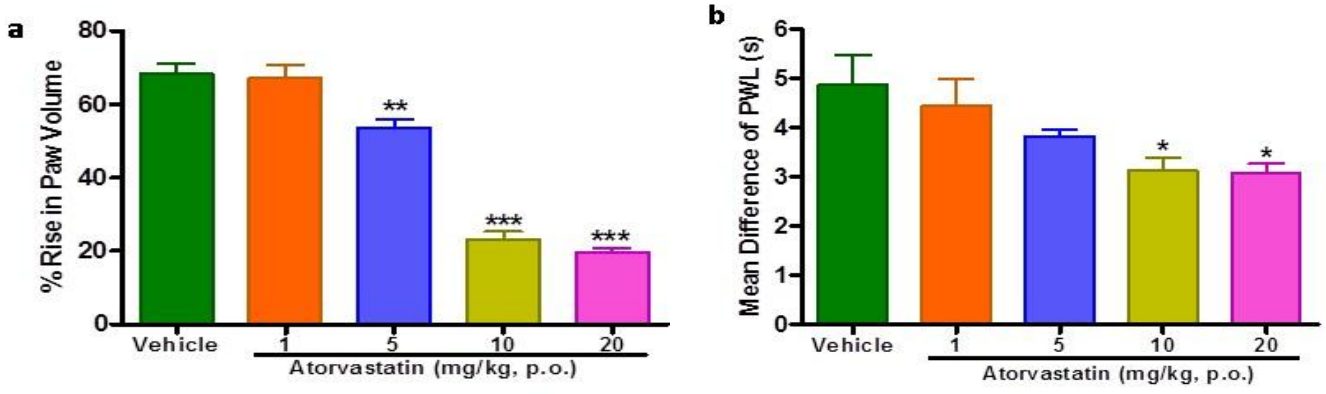
Graph shows the mean±SEM of the PWL of vehicle and 2% carrageenan treated group post-carrageenan injection. *p<0.05; **p<0.01; ***p<0.001; paired *student's t-test* comparing PWL of vehicle v/s 2% carrageenan treated group at same time point.

Fig 3. Dose response of milnacipran in carrageenan-induced inflammation and inflammatory pain model



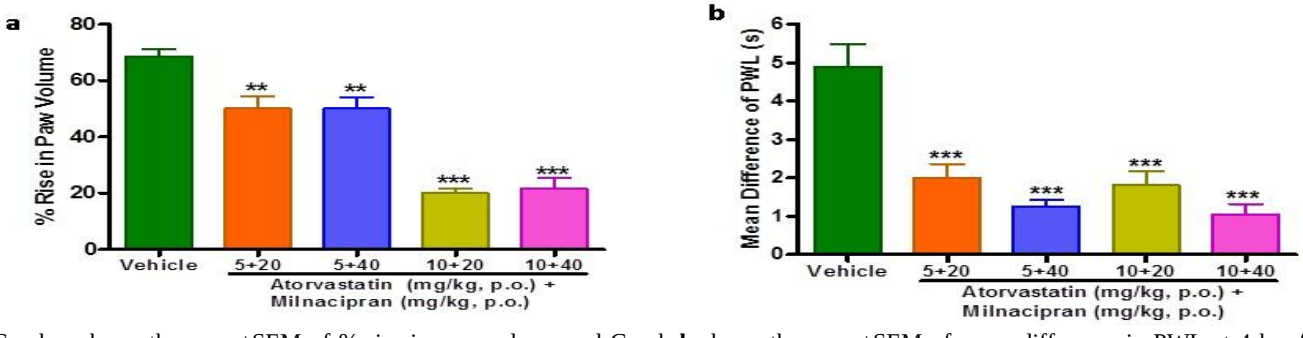
Graph **a** shows the mean±SEM of % rise in paw volume and Graph **b** shows the mean±SEM of mean difference in PWL at 4 hr after carrageenan injection. **p*<0.05; ***p*<0.01; ****p*<0.001; one-way ANOVA followed by Tukey's multiple comparison test as post hoc analysis comparing to vehicle treated control group.

Fig 4. Dose response of atorvastatin in carrageenan-induced inflammation and inflammatory pain model



Graph **a** shows the mean±SEM of % rise in paw volume and Graph **b** shows the mean±SEM of PWL at 4 hr after carrageenan injection. **p*<0.05; ***p*<0.01; ****p*<0.001; one-way ANOVA followed by Tukey's multiple comparison test as post hoc analysis comparing to vehicle treated control group.

Fig 5. Anti-inflammatory and analgesic effect of combination of different doses of atorvastatin and milnacipran in carrageenan-induced inflammation and inflammatory pain model



Graph **a** shows the mean±SEM of % rise in paw volume and Graph **b** shows the mean±SEM of mean difference in PWL at 4 hr after carrageenan injection. ***p*<0.01; ****p*<0.001; one-way ANOVA followed by Tukey's multiple comparison test as post hoc analysis comparing to vehicle treated control group.

DISCUSSION

Carrageenan-induce inflammation and hyperalgesia is a well-established model of inflammation. Carrageenans are linear sulfated polysaccharides derived from red seaweeds, which possess strong inflammatory properties. Winter et al. first used carrageenan injections into the plantar surface of the rodent paw as an edema-based assay for evaluating anti-inflammatory drugs (Winter *et al.*, 1962). Subsequently, this model has been used widely to evoke thermal hyperalgesia in rodents (Hargreaves *et al.*, 1988; Morris, 2003). Following carrageenan injection, edema and behavioral hypersenstitivity are established by 4 hr after injection and last through at least 96 hr (Morris, 2003). The results of the present study are in line with the previous work. In this study the maximum percentage rise in paw volume and maximum reduction in paw withdrawal latency in ipsilateral paw was observed at 4 hr after carrageenan injection. The edema was decreased after 4 hr but the thermal hyperalgesia persists till 24 hr after carrageenan injection.

Earlier studies suggest that persistent inflammation leads to the hyper-excitability of dorsal horn neurons within the spinal cord, also known as central sensitization (Dubner and Ruda, 1992; Mannion and Woolf, 2000). Central sensitization is characterized by altered responsiveness of dorsal horn neurons, expansion of receptive fields and plasticity of neuronal connections within the pain transmitting pathways leading to increased neuronal activity at supraspinal sites and to dysfunction of the endogenous spinal and supraspinal pain inhibitory mechanisms (Mannion and Woolf, 2000; Urban and Gebhart, 1999). An imbalance of the excitatory and inhibitory mechanisms within both the ascending and descending pain inhibitory pathways could ultimately lead to persistent pain (Urban and Gebhart, 1999; Ren and Dubner, 2002). In one of the recent clinical trial, it is proven that milnacipran is a safe, well tolerated and effective treatment of multiple symptoms of fibromyalgia (Mease *et al.*, 2009). Thus, using 5-HT and NE reuptake inhibitor like milnacipran to restore this balance could be beneficial in persistent pain conditions. In the present study milnacipran attenuated the nociceptive behavior of carrageenan significantly at the doses of 20, 40 and 60 mg/kg. It increases the paw withdrawal latency to thermal stimuli significantly. Milnacipran failed to produce anti-inflammatory activity as it did not inhibit the rise of paw volume after carrageenan injection.

Statins have not been evaluated for their selective analgesic action in commonly used experimental models of analgesia but has been evaluated in some of the models of inflammatory pain. As reported by Barsante *et al*., atorvastatin attenuated the hypernociception in inflamed joints in rat model of adjuvant-induced arthritis (Barsante *et al.*, 2005). Also in a model of mechanical hypernociception in mouse paw with an electronic pressure-meter, atorvastatin inhibited the inflammatory hypernociception after oral administration (Santodomingo-Garzo´ *et al.*, 2006). It has been reported that pretreatment with atorvastatin reduces the levels of bradykinin, tumor necrosis factor-a (TNF-α), interleukin-1b (IL-1b) the chemokines which are involved in the induction of hypernociception (Santodomingo-Garzo´ *et al.*, 2006). Some of the recent studies supported the anti-nociceptive effect of atorvastatin in various animal model of pain in rat and mice (Swapnil and Smita, 2012; Ghaisas *et al*., 2010; Dwajani *et al.*, 2012). In the present study atorvastatin significantly inhibited the rise of paw volume induced by carrageenan injection. Atorvastatin at the dose levels of 5, 10 and 20 mg/kg, showed dose-dependent anti-inflammatory activity in this model of acute inflammation. Also, atorvastatin produced significant anti-nociceptive effect. Only higher dose of atorvastatin (10 and 20 mg/kg) increased the paw withdrawal latency to thermal stimuli in this model of hyperalgesia. In the combination study, milnacipran did not potentiate the anti-inflammatory activity of atorvastatin at any dose level. In contrast to that, both doses of atorvastatin potentiate the anti-nociceptive activity of milnacipran. Though it is not producing any additive effect when administered along with milnacipran but it inhibited the thermal hyperalgesia more significantly rather than when treated with milnacipran alone. Further studies are required for in-depth knowledge of mechanism by which the atorvastatin potentiates the anti-nociceptive behavior of milnacipran.

CONCLUSION

Milnacipran showed significant dose-dependent anti-nociceptive effect in carrageenan-induced inflammatory pain model. Milnacipran decreased the mean difference of the paw withdrawal latency to thermal stimuli however; it failed to exhibit any anti-inflammatory activity as it did not inhibit the rise of paw volume. Atorvastatin produced anti-inflammatory effect in dose-dependent manner. At higher doses atorvastatin showed significant anti-nociceptive effect. All four combinations of atorvastatin and milnacipran showed statistically significant reduction in rise of paw volume as well as mean difference of paw withdrawal latency. The atorvastatin produced the additive effect by potentiating the anti-nociceptive effect of milnacipran in this model of carrageenan-induced inflammation and hyperalgesia however, milnacipran failed to potentiate the anti-inflammatory activity of atorvastatin.

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